BACTERIOLOGICAL REVIEWS

VOLUME 5

BALTIMORE, MD
1941

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JOHNSON REPRINT CORPORATION
111 Fifth Avenue, New York, N Y 10003

JOHNSON REPRINT COMPANY LTD
Berkeley Square House, London, W 1

First reprinting, 1967, Johnson Reprint Corporation
Printed in the United States of America

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ERRATA

In Vol 4, page 157, line 14 should read
"The organisms contain the decarboxylases only if they "

Page 159, line 8 should read
"and 2 produced putrescine from ornithine "

THE FILTRABLE MICROÖRGANISMS OF THE PLEUROPNEUMONIA GROUP

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In 1898, when our knowledge of filtrable viruses was but a few years old, the then ultramicroscopic etiological agent of bovine pleuropneumonia was cultivated in a cell-free medium by Nocard, *et al* (63) The morphologic studies of Bordet (3) and of Borrel, *et al* (4) in 1910, and of others later on established the remarkable polymorphic character of the microörganism, and the filtration experiments through graded collodion membranes carried out by Elford (32) in 1929 established that the cultures contained "particles," 125 to 150 mμ in size, which were capable of reproducing not only themselves but also the larger and more complex structures For 25 years after its cultivation in serum broth it was unique among infectious agents, but was practically always grouped with the filtrable viruses In 1923, Bridré and Dona-

tien (5) demonstrated not only that the filtrable etiological agent of agalactia of sheep and goats could be cultivated *in vitro* in the same manner as the microorganism, or virus as it was still called, of bovine pleuropneumonia but also that, excepting the pathogenic and immunologic properties, there was the closest morphological and biological resemblance between these two agents (6) Although in the ensuing period many communications appeared dealing with the morphology and mode of reproduction of the bovine pleuropneumonia and agalactia microörganisms (43, 53, 65, 66, 103), another 11 years passed before a new member of this group was discovered In 1934, Shoetensack (86) reported the cultivation in cell-free media of morphologically and biologically similar microörganisms from dogs suffering of distemper, and although insufficient work was done on the relation of these microörganisms to the virus of canine distemper, it was nevertheless established that members of the pleuropneumonia group occurred in dogs

In 1935, Klieneberger (44) reported the remarkable observation that a pleuropneumonia-like microörganism could be demonstrated in all available strains of *Streptobacillus moniliformis*, a gram-negative pleomorphic bacillus which is a normal inhabitant of the nasopharynx of rats and the cause of at least one type of rat-bite fever in man Although she has been able to isolate this microörganism (L_1) in pure culture and maintain it in continuous subculture without reversion to *Streptobacillus moniliformis*, other investigators (21, 24) have challenged her hypothesis that such symbiosis existed and suggested that the L_1 microörganism is a variant of the bacillus These studies, however, have led to the isolation of other pleuropneumonia-like, pathogenic microörganisms from rats, unassociated with bacteria and distinct from L_1 (47, 48)

The next important contribution in this field came in 1936, when Laidlaw and Elford (52) reported on a new group of filtrable microörganisms which they isolated from raw sewage These resembled the other members of the pleuropneumonia group in that minute particles, 125 to 175 mμ in size, reproduced the same

type of polymorphic structures, and gave rise to similar microscopic colonies on solid media, but differed in not requiring protein for their growth, also they possessed no pathogenic properties Seiffert (84) confirmed these observations when he reported the isolation of similar microörganisms from filtrates of soil, compost, decomposing leaves, and manure

Late in 1938, Sabin (75) and Findlay, *et al* (35) simultaneously described the isolation of a new pleuropneumonia-like microörganism from mice which developed a peculiar nervous disease in the course of routine passage of toxoplasma or lymphocytic choriomeningitis virus The studies on this group received a new orientation when it was demonstrated (74, 75, 76) that this filtrable microorganism of the mouse, although capable of multiplying in a cell-free medium *in vitro*, was an intracellular parasite *in vivo*, with a special affinity for the mesenchymal cells of the pleura, peritoneum, and joints, and that during the course of its multiplication a typical neurotropic exotoxin was produced which gave rise to choreiform nervous signs Furthermore, early in 1939 Sabin (76) isolated from normal mice another such microorganism, immunologically distinct from the first, and with such limited cellular affinities that it could multiply only in the mesenchymal cells of the joints in which it produced a proliferative, progressive, and chronic ankylosing arthritis Subsequent studies (77, 80) revealed that normal mice are carriers of these pathogenic microörganisms, especially in their conjunctiva and nasal mucosa, and at least five immunologically distinct types have already been described (80) Although attempts to demonstrate members of the pleuropneumonia group in pathological material from patients with rheumatic fever and rheumatoid arthritis have met with no success, studies are being continued on human beings (80), and Dienes (28) has already brought forth more than suggestive evidence of their existence in the female genital tract

At the present time our knowledge has progressed far enough to indicate that there exists in nature a distinct group of filtrable, saprophytic and parasitic microörganisms, of which the etiological

agent of bovine pleuropneumonia is the prototype, and which possess properties that clearly distinguish them from the ordinary bacteria, the filtrable viruses, and the rickettsiae

GENERAL CHARACTERISTICS OF THE GROUP AND METHODS OF STUDY

Criteria for Identification The criteria which admit a microorganism into the pleuropneumonia group are (1) growth in cell-free culture media with the development of polymorphic structures including, "rings," globules, filaments, and minute, filtrable elementary bodies, usually 125 to 250 mμ in size, which are the minimal reproductive units, and (2) the development on suitable solid media of characteristic minute colonies which may be as small as 10 to 20μ and as a rule not larger than 600μ These characteristics are shared by the saprophytic as well as the parasitic members of the group, but the latter are further distinguished by their inability to grow in cultures that do not contain a high concentration of serum protein

Cultivation from Infected Tissues and Exudates When smears, made with animal tissues or exudates in which a pathogenic member of the group has multiplied, are stained with the ordinary aniline dyes or by Gram's method one can find no formed elements suggesting the presence of a microorganism Furthermore, when such tissues or exudates are cultured on ordinary solid or fluid media or on media containing less than 5 to 10 per cent of blood or serum, there is usually no growth It is for this reason that a number of filtrable infectious agents were believed to be viruses until cultivation on suitable media revealed that they were members of the pleuropneumonia group However, even when suitable media are employed the primary growth, because it can be so unlike that which occurs with the familiar bacteria, may fail to be recognized by the uninitiated The media usually considered suitable consist of heart-muscle infusion peptone broth or agar (2 per cent) having a pH of 7 6 to 8 0, to which is added 10, 20, 30, or even 40 per cent of various animal serums (horse, bovine, rabbit) or human ascitic fluid Boiled blood has been incorporated in the basic medium by some (24, 45, 86) and glucose by others (75) because primary isolation of certain strains is thus

facilitated The use of dried meat extracts instead of fresh meat infusion was enough on one occasion in my experience to make the difference between growth and no growth

When a suitable fluid medium is inoculated with infected tissue or exudate which in itself gives rise to appreciable turbidity, the best procedure to follow is subculture of 0 1 to 0 2 ml into fresh medium on the 4th day and again on the 7th day If the initial culture is not clouded by the inoculum the first sign of growth may be the development of very slight diffuse turbidity, which can be appreciated only by comparison with an uninoculated tube of medium, or by the appearance of a slight granular sediment An uninoculated tube of the culture medium is, therefore, always incubated along with the inoculated ones In primary cultures the first turbidity or other evidence of growth may not appear for 3 to 14 days Subculture should be carried out as soon as growth is suspected, or on the 4th and 7th days if the medium remains clear even if a Giemsa-stained film reveals no formed elements of any kind I have referred to the latter type of subculture as "blind passage" (75, 76), because good growth developed on a number of occasions in such subcultures even when the primary culture itself remained negative over a period of weeks After several serial passages have been carried out growth may become apparent as early as 24 or 48 hours That the transmissible turbidity which appears in fluid cultures is due to the growth of a member of the pleuropneumonia group can be proved in the following ways (a) gram-stained smears reveal no ordinary bacteria and indeed may show no distinct forms of any kind, (in smears of centrifuged sediments suspended in saline, the microorganisms, though faintly stained are gram-negative), (b) Giemsa-stained smears reveal characteristic polymorphic structures, (c) demonstration of *large numbers* of similar polymorphic structures in the dark-field, and most important of all, (d) development of characteristic microscopic colonies when some of the fluid culture is seeded on solid medium containing the same kind of protein In doubtful cases, the dark-field examination may be most misleading since structures which morphologically are remarkably similar to these microörganisms may be

found in uninoculated tubes especially those incubated at 37° for a long time These "pseudo" structures, however, fail to show up in Giemsa-stained films and no colonies appear on solid media

The use of 30 per cent serum or ascitic fluid agar for primary isolation of these microörganisms from infected tissues or exudate often gives decisive results more quickly, and when the material to be cultured is contaminated with ordinary bacteria, it is the method of choice The tissue is minced to expose a greater surface which is brought into contact with the soft agar in many different places, if the material to be cultured is fluid, 0 1 ml of it is poured on and allowed to spread over the agar The optimum colonial development occurs when evaporation of the medium is prevented, which is accomplished by inserting a piece of filter paper in the cover and sealing the Petri dish with parafilm (a procedure suggested to me by Dr Homer Swift) Macroscopic examination of such an agar plate may reveal nothing but the dried inoculum to the uninitiated and oftentimes to the experienced as well With the aid of a hand lens, however, and sometimes with the naked eye it is possible to discern the minute colonies which may require as little as 2 days' or as much as 7 days' incubation to become apparent These colonies are best examined under the microscope, with the substage condenser removed, using the 10 × ocular and 16 mm objective with oblique illumination obtained from a blue light by the concave mirror Where the growth is not confluent, the isolated colonies appear distinctly outlined and slightly elevated, with a nipple-like darker center or surface vacuolar meshwork, and 10 to 600μ in size depending on the species and conditions of growth When such colonies are present or suspected, the piece of agar on which they occur is cut out and streaked on another agar plate which is incubated as before Numerous minute colonies usually appear in a few days along the streaks if microörganisms of the pleuropneumonia group are present The "pseudo colonies" occurring on certain kinds of serum agar described by Brown, Swift, and Watson (8) are sufficiently different from those of the pleuropneumonia group not to cause confusion when their possible occurrence is appreciated Once colonial growth is established on

solid medium, and a Gram stain of a film of such colonies reveals no ordinary bacteria, it is advisable to establish growth in fluid media by dropping a piece of agar with many such colonies into a tube of fluid medium containing the same kind of serum or ascitic fluid, whichever may have been present in the solid medium It may take a few days before growth appears and when it does it is usually in the form of granules or flakes close to the piece of agar, and only rarely as a diffuse turbidity After a number of rapid subcultures (sometimes as many as 6 to 10 are required) a culture often changes from granular suspension to diffuse turbidity When all colonies do not appear the same, it is advisable to subculture single colonies on solid media In this manner Sabin and Johnson (80) were able to demonstrate three distinct immunological types in a culture from the nasal mucosa of a single mouse

When one has thus obtained characteristic growth on fluid and solid media, it is desirable to demonstrate filtrability preferably through suitable gradocol membranes before finally classifying a microorganism as a member of the pleuropneumonia group Filtration through Berkefeld filters, impervious to *Serratia marcescens*, is significant only when it is shown by plating or dilution that a relatively large number of reproductive units, although not necessarily a large proportion of the total, have passed through

General Remarks about Conditions of Growth The saprophytic members of the pleuropneumonia group differ from the parasitic ones in that they do not require protein for growth and can multiply at 22° Although Nocard, *et al* (63) first cultivated the microorganism of bovine pleuropneumonia in a medium containing 4 to 5 per cent of serum, practically all the other members of this group require 10 per cent or more of serum for primary isolation After adaptation to growth *in vitro*, multiplication also occurs with smaller concentrations of serum protein Experiments with the microorganism of agalactia (6) revealed that when the concentration of serum is increased to 80 or 90 per cent growth is retarded, and in pure serum it is apparently completely inhibited, no growth whatever occurred in serum diluted with physi-

ologic salt solution In the case of bovine pleuropneumonia growth was reported to be entirely arrested when 50 per cent of the medium consisted of horse serum (94) Although the function of the protein is still unknown I have observed in working with the microorganisms isolated from mice that some strains and types can become so thoroughly adapted to the protein of one species that they fail to grow or will grow very poorly when transferred to a culture medium containing the serum of another species

Growth occurs both aerobically and anaerobically, but, with the exception of the microörganisms isolated from dogs, it is less abundant under anaerobic conditions Whenever the influence of pH has been studied it was found that pH 7 8 to 8 0 is optimum for growth The addition of various sugars improves growth in some instances and not in others In the case of agalactia, the addition of glucose, levulose, galactose, raffinose, arabinose, xylose, sucrose and maltose in concentrations of 1 to 2 per cent is reported (6) as exerting a retarding effect while lactose and mannitol, for example, have a stimulating effect Fermentation often occurs with the production of acid, and when a pH of 7 0 or less is reached growth usually ceases With the mouse microorganisms I found that in the presence of 0 5 per cent glucose, subculture is no longer possible on the 3rd day of fully adapted cultures, while without the added sugar positive subcultures can be obtained even at the end of a month at 37° This amount of glucose, however, did not have the same effect on at least one rat strain (L_4) and not quite as rapid an effect on the microörganism of bovine pleuropneumonia

Reduction of hemoglobin has been observed in cultures of the pleuropneumonia microörganisms, and in tests carried out with Doctor Joel Warren both hemolysis and reduction of hemoglobin were observed on solid media with the pleuropneumonia, L_3, and Types A, B, and C mouse microorganisms but not with L_1 and L_4 I have also observed that with some strains the yellow pigment of the added serum disappears with the first signs of growth Certain metabolic studies on the microorganisms of the group have been reported (41, 71, 72, 98)

Preservation of Cultures Bridré and Donatien (6) reported that the microörganism of agalactia under aerobic conditions at 37° lost its reproductive capacity at the end of a month (presence or absence of added sugar not indicated) Under anaerobic conditions, however, or when the fluid culture was covered with vaseline after aerobic cultivation, it was still possible to obtain positive subcultures after a sojourn of 22 months at 37°, cultures that were similarly sealed and stored at 0°, 6° to 12°, and 25° did not survive as long, failing to yield growth even after 5 months In my own work when it was desirable to carry cultures with sugar added to the medium, they would be stored in the refrigerator and subcultures made at 10- to 14-day intervals, although with some strains it was still possible to obtain growth after 5 to 6 weeks Cultures on solid media kept in plates or tubes sealed with parafilm may be subcultured at monthly intervals In order to preserve various strains or types for future studies and before their pathogenic or other properties have been changed by too many subcultures, Swift's method for preserving bacteria has been applied (92) Twenty-five ml or more of full-grown culture is spun at 4000–5000 r p m for one hour in an angle centrifuge Approximately 10 per cent of the supernatant liquid is left behind and used for resuspending the sediment This concentrated suspension is distributed in 0 1 ml amounts in small cotton-plugged tubes, which are then rapidly frozen with solid CO_2, and put into a chilled desiccator containing a large dish of P_2O_5 The desiccator is placed in an insulated container over a tin box containing solid CO_2 so that it remains at a temperature of approximately $-10°$ to $-20°$ during the entire period of evacuation and drying which usually does not require more than 24 hours Suitably dried specimens appear as a white or slightly yellowish bit of foam The tubes are sealed with picein and stored at room temperature to prevent the seal from cracking To reconstitute, a small amount of medium is added to the dried material and transferred to a tube or flask of fresh medium Cultures dried in this manner have yielded positive subcultures after more than a year of storage Drying of cultures from the frozen state on the Mudd-Flosdorf

apparatus has for some reason been unsuccessful on a number of occasions, on the other hand, I have had no difficulty in preserving the same microörganisms in infected tissue dried with this apparatus

Morphology and Mode of Reproduction The existing descriptions of the morphology and mode of reproduction (3, 4, 25, 43, 53, 65, 66, 94, 96, 103) have varied a good deal depending on (a) whether the investigator followed the growth on solid or liquid media, (b) the type or age of culture used, and (c) the method of examination, i e stained films, agar fixation, or dark-field It is remarkable how different is the impression gained of the morphology of one of these microörganisms from an examination of preparations of colonies on solid media, and of stained films or dark-field preparations of growth on liquid media Quite aside from the recognized fact that the growth units may be so plastic as to undergo considerable distortion in films and smears, the usual preparation from a colony on solid medium presents such large discs, globules, and even amorphous masses with chromatic bodies, that the general reaction of utter confusion is not limited to the uninitiated Near the edge of such a preparation one can find structures which are similar to those seen in liquid media While not denying that studies of growth on solid media are essential for the ultimate concept of the true mode or modes of reproduction, I personally find it much less confusing and definitely more decisive for comparative purposes to study the growth on fluid media either stained or with the dark field By these methods one can find morphological differences even among different types found in the same species of animal For example, the Type A microorganism of the mouse exhibits the elementary-body-like granules, small bacilliform or spirillar forms during the early phases of growth, the rings, triangles, quadrangles, etc with the denser bodies distributed irregularly through these structures, but at no time have streaming filaments attached to these structures been found such as are regularly seen in the Type B microorganism and in certain other members of the group

While various investigators have described many distinct types

of reproduction, the following synthesis by Ledingham (53) for the microörganisms of pleuropneumonia bovis and agalactia corresponds most to my own observations on many other members of this group with certain allowances for individual differences

"Commencing with the filterable viable element we note its spore-like capacity to pullulate to filamentous and ramifying elements The protoplasmic substance of these filaments, whether at their extremities or in their course, retains the power to elaborate the more deeply stained chromatic and consolidated nodes from which further moniliform growth proceeds The term 'moniliform' I use only for convenience to express the beaded character of the growing filament Unlike those of a moniliform streptobacillus the "beads" exhibit the greatest variety in size and shape, particularly during the early stages in their development In early colonial growths, as I have described and figured, these chromatic condensations may assume considerable dimensions, bizarre shapes and a quite characteristic differential reaction of their outer and inner parts to the Giemsa stain, and their further pullulation by a unipolar or multipolar pseudopodial budding process furnishes the great mass of polymorphic units, rings, spheres, filaments, etc present in cultures at the period of maximal growth "

Growth on Chorioallantoic Membrane and in Tissue Culture Tang, *et al* (95) inoculated 9- to 11-day-old eggs with a culture of pleuropneumonia bovis "Oedema and sometimes white spots" were found throughout the chorioallantoic membrane Although the embryos were usually dead 3 or 4 days after inoculation, positive cultures were obtained only from the chorioallantoic membrane and from the surface of the embryo but not from the internal organs, yolk or amniotic fluid While they saw all the usual forms of the microörganism on dark-field examination, they found no cytoplasmic or intranuclear inclusions in Giemsa-stained scrapings from affected areas of the chorioallantoic membrane Sullivan and Dienes (91) working with microörganisms isolated from mice (some of which I identified as Type A) reported that they were unsuccessful in obtaining growth on the chorioallantoic membranes of chick embryos in the usual manner, but by chilling the embryo to death at 4° prior to incubation they were able to obtain growth and serial passage Swift (personal

communication) observed that the Type A microörganism of mice is the only one that grows not at all or very feebly on living chorioallantoic membranes of chick embryos, while the Type B microorganism, certain rat strains, and pleuropneumonia grow well, in all instances, however, growth was better with embryos that were killed by chilling I was able to obtain growth and serial passage of the Type B microörganism in a medium consisting of 0 1 ml of minced mouse embryo tissue and 4 5 ml Tyrode's solution with no added serum The multiplication in this medium appeared to be predominantly intracellular Sullivan and Dienes (91) using mouse microörganisms demonstrated growth on a minced-chick-embryo-tyrode solution medium as well as on embryo-tyrode-agar (Zinsser, *et al* 107)

Thermal Death Point While some members of the pleuropneumonia group are killed by relatively low temperatures, the thermal death points of others are in the same range as those of most viruses and bacteria Thus the Type A microorganism of mice is killed by a temperature of 45° maintained for 15 minutes (74, 75), while other types isolated from mice (31) are not affected by 45° or 50° in 15 minutes but are killed at these temperatures in 30 minutes, or at 55° in 15 minutes Laidlaw and Elford (52) reported that the majority of "sewage microörganisms" were killed at 45° in 15 minutes and that none withstood 55° for 5 minutes The microorganism of agalactia on the other hand is reported (6) to resist 50° for 1½ hours, and 53° for 7½ minutes but not for 10 minutes The microorganisms of the dog are killed at 48° in 30 minutes, 50° in 10 to 20 minutes, and 55° in 5 minutes (87) The L_1 microorganism associated with *Streptobacillus moniliformis* resists 53° for 15 minutes (45) The filtrable agent of spontaneous polyarthritis of rats (subsequently identified (1, 49) as belonging to the pleuropneumonia group) is killed by 40° in 4 hours, 42° in 60 to 75 minutes, 44° in 30 minutes, 45° in 10 minutes, 46° in 6 minutes, 48° in 4 minutes, and 50° in 2 minutes (Collier, (15)) Collier's data are given here in detail because they are extensive enough to indicate the curve of denaturation of the viable material In contrast to this marked lability, one may note that the filtrable pyogenic agent

from rat sarcomas (identical with the L_4 microörganism of the rat pleuropneumonia group) was reported (100, 101) to be only attenuated at 56° in 30 to 60 minutes and completely inactivated after one hour at 60° Another strain of the L_4 microorganism (at first called L_7) isolated from the joints of rats with spontaneous polyarthritis was reported to be killed by heating at 50° for 30 minutes (37) Whether or not this means that different strains of the same serological type can have distinct thermal death points, is, of course, problematical

Virulence of Cultures The pathogenic properties of cultures of the various microörganisms will be discussed later on, but it can be stated here that cultures of some of the members of this group lose their virulence after but a few passages *in vitro* while others remain pathogenic for more than a hundred passages Furthermore, the medium on which a given strain is grown may also modify its pathogenicity (30) While Findlay, *et al* (37) stated that "it seems that all pleuropneumonia-like organisms require an adjuvant such as agar or cells to start infection, if injected into animals", that has not been found necessary with the microorganisms of bovine pleuropneumonia, agalactia, and those I isolated from mice

Bovine Pleuropneumonia and Properties of the Etiological Agent

Bovine pleuropneumonia is a highly contagious disease of cattle characterized by extensive consolidation, pleurisy, and subpleural effusion affecting usually one and sometimes both lungs In young calves there is occasionally also joint involvement The disease, one of great economic importance, has been recognized in Europe for over 200 years, and at present is distributed throughout the world with the exception of North America, Western Europe, and India (97) It appeared in the U S A in 1843 but was finally eradicated The infectious agent was early shown to be present in great concentration in the serous exudate or lymph of the lung by subcutaneous inoculation into other bovines which develop after an incubation period of 8 to 15 days an extensive edema spreading from the site of inoculation Cattle, so inoculated, have fever and often die but never develop

the lung lesions so characteristic of the natural disease or any other lesion[1] excepting that directly associated with the site of inoculation, recovered animals, however, are immune not only to reinoculation but also to the natural disease Although it is generally assumed that in nature the disease is transmitted by droplet infection through the respiratory tract, it is practically impossible to reproduce it experimentally by this route, even after intratracheal injection of highly virulent lymph, or by any other route In a renewed attempt to produce pleuropneumonia by intratracheal inoculation, Daubney (20) succeeded only once in 22 trials with highly virulent lymph In view of the highly contagious character of the disease one wonders if the infectious agent may not have to be carried into the lung tissues by some parasite, a possibility which does not seem to have been investigated thus far, and which might well repay investigation particularly in view of Shope's recent observations on the virus of swine influenza (89)

The infectious agent, which was early shown to pass through filters retaining the known bacteria (29), could not be grown by ordinary procedures or demonstrated in stained preparations of the pathological exudates and tissues, until Nocard and Roux in collaboration with their students (63) adopted the collodion sac technique previously used by Metchnikoff, *et al* (57) in studies on cholera The collodion sacs, containing ordinary broth and a "trace" of virulent lymph, were sealed and implanted into the peritoneal cavity of rabbits and guinea-pigs After 15 to 20 days

[1] An observation by K F Meyer (58a) is of great interest with respect to the predilection of so many members of the pleuro-pneumonia group for the joints He reported "Lung-sickness virus collected from a sick animal and used for prophylactic inoculations [tail] in cattle, produced in animals of different ages a polyarthritis serofibrinosa This phenomenon was not due to individual disposition, as is occasionally observed in calves, but it was specific for the strain of virus used by us The subcutaneous inoculations of synovial liquid of animals affected by this particular strain produced, besides the typical local reaction, a poly-arthritis in all animals [bovines] experimented on The synovial liquid represented a pure virus, and the micro-organism of pleuro-pneumonia could be cultivated from it The specific action of the strain became lost in subcultures or by passage through an animal, and no secondary joint affections could be produced subsequently by inoculation of cultures "

growth was present in the sacs implanted in the rabbits but not in the guinea-pigs Serial passage was accomplished and the cultures were proved to be virulent on subcutaneous injection in cattle which upon recovery were immune to reinoculation with virulent lymph They soon discovered, however, that this laborious technique was unnecessary, and that they could obtain growth *in vitro* by using an especially rich peptone medium to which was added a small amount of bovine or rabbit serum They were also first to make use of a step which all new investigators, who find themselves studying a member of the pleuropneumonia group, sooner or later discover for themselves, that the ordinary criteria used for judging growth of bacteria may not suffice, they said "La culture du microbe de la péripneumonie est abondante, pourtant, elle ne provoque qu'un très léger louche, une opalescence à peine sensible du liquide, *on est obligé, pour se convaincre de la réalité de la culture, d'examiner comparativement, à côté d'elle, un tube de meme bouillon non ensemencé*" [My italics] Subsequent work by Dujardin-Beaumetz (29) established its growth on solid media, and that of Bordet (3) followed by Borrel, *et al* (4) revealed for the first time the complex morphology of the infectious agent on artificial media It was Borrel and his coworkers (4) who first proposed the name of "*Asterococcus mycoides*" for this microörganism because it recalls "les principaux caractères de ce microbe intéressant, gaine muqueuse, filaments pseudo-mycéliens, polarités multiples", and it is of interest to quote their prophecy, which took 13 years to come true, namely "Il est difficile en l'état actuel de le comparer à d'autres types puisqu'il est le seul connu de son espèce, mais on peut déjà prévoir qu'il ne restera pas isolé dans ce groupe "

A strain of pleuropneumonia bovis which has grown *in vitro* for some time imparts a distinct turbidity to the fluid medium which upon shaking exhibits silk-like whorls, that are not seen with any other member of the group described thus far The tendency to form long chains of rings may be responsible for this property Tang, *et al* (94) working with strains isolated in China indicated that no growth was observed in plain broth, blood broth, litmus milk, blood agar, Loeffler's serum and Bordet-Gengou's

medium They found that "hormone" broth with 2 per cent peptone and 10 per cent horse serum was the best Certain carbohydrates are fermented with the production of acid but not gas "Glucose, fructose, mannose, maltose and dextrin were strongly fermented, sucrose and trehalose only slightly attacked, while raffinose, inulin, galactose, salicin, xylose, mannitol, arabinose, amygdalin, lactose, dulcitol, iso-dulcitol, sorbitol, inositol, erythritol and adonitol were not acted upon" They also stated that the microörganism was bile-soluble, it reduced hemoglobin in fluid cultures when freshly isolated and for a certain number of passages thereafter, but that old strains lost this property Five minutes' contact with anaesthetic ether was enough to kill it

As regards the host range of the infectious agent of pleuropneumonia bovis, Willems (99) established in 1850 that material which infected cattle was innocuous for the goat, sheep, dog, swine, rabbit, guinea-pig, poultry and man Dujardin-Beaumetz (30) reported that while cultures in bovine-serum broth had the same limited host range as the original lymph, the same microorganisms grown in cultures containing sheep or horse serum, were highly infectious for sheep and goats In these animals the cultures produced not only the marked swelling at the site of subcutaneous infection but on occasion also fever, polyarthritis, and death With cultures, presumably grown in horse-serum broth, Tang, *et al* (94) were also able to infect goats and one water buffalo, but not "white mice, hamsters, albino rats, guinea-pigs, rabbits and cats after subcutaneous, intraperitoneal, intracerebral, intravenous and in some cases intratesticular inoculation" [See also Walker (97)]

Agalactia of Sheep and Goats and Properties of the Etiologic Agent

Agalactia receives its name from the manifestation which first drew attention to the disease, but careful clinical and experimental studies have established that it is a systemic disease, affecting males and females alike, with particular involvement of the joints, the eyes, and in lactating sheep and goats the mammary glands (9) Agalactia has been predominantly a European disease,

being especially prevalent in the mountainous regions of Italy, France, and Switzerland, in recent years it has occurred in Algeria. The course of the disease may be acute or chronic. While death may ensue before the appearance of the usual lesions, that does not occur often and the animals exhibit arthritis, keratitis and occasionally a vesiculo-pustular skin eruption, the lactating females develop mastitis and the scrotum may be inflamed in the males. The infected mammary glands stop secreting milk, develop many indurated nodules, and atrophy. The joints usually affected are those of the carpus and tarsus and less often the femoro-tibial, humero-radial, coxo-femoral and metacarpals. In most animals the arthritis clears up, while in some the process occasionally goes on to abscess-formation with involvement of the articulating surfaces and ultimate ankylosis. In the chronic form of the disease there may be remissions and exacerbations of the arthritis without ultimate deformity. There is usually generalized wasting of the musculature. The pregnant animals abort giving birth to dead fetuses or monsters.

The etiological agent has been shown to be filtrable (9, 10). It is present in the secretions of the infected mammary glands, eyes, and joints, and in the early stages of the disease in the blood. Intravenous inoculation or even feeding of infectious material can produce the typical disease with localization in the mammary glands, joints and eyes, indicating that the agent has a special affinity for these tissues. In 1923, Bridré and Donatien (5) first cultivated the infectious agent (from the fluid of an affected joint) in broth containing 20 to 30 per cent of horse serum. The cultures so obtained and others grown subsequently from infected milk or lymph nodes reproduced the disease in sheep and goats. The cultures were shown to be infective by the cutaneous, subcutaneous, intra-articular, and intravenous routes. Bridré and Donatien recognized the close morphological, cultural, and biological resemblance of their microorganism to that of pleuropneumonia bovis, but were able to show that the two were different in their pathogenic properties and that there was no serological relationship or cross immunity between them (6).

The microorganism of agalactia grows in cows' and goats'

milk without producing any obvious change in the medium, while at least one strain of bovine pleuropneumonia microörganism tested by Bridré and Donatien (6) failed to grow in milk Some sugars like glucose, levulose, galactose, raffinose, arabinose, xylose, sucrose, and maltose retard growth while others like lactose, mannitol and erythritol favor it Good growth occurs in the presence of staphylococci and certain other bacteria and when these organisms are encountered in the first cultures of infectious material, one need only remove them by filtration through a suitable Berkefeld "V" or Chamberland L_1 candle to obtain a pure culture of the agalactia agent

Pleuropneumonia-like Microörganisms of Dogs and Their Relation to Canine Distemper

In 1934, Shoetensack (86), working in Japan, reported the isolation of pleuropneumonia-like microorganisms from material obtained from dogs with distemper His own illustrations and the subsequent work done on his strains by Klieneberger (47) leave no doubt that the filtrable microorganisms he called *Asterococcus canis* are indeed, as he himself indicated, members of the pleuropneumonia group Their role in the etiology of canine distemper is, however, another matter At first he cultivated these microorganisms from the purulent nasal secretions, and inoculated one-half of an agar slant of a 4-day, 7th generation culture subcutaneously into 4 puppies, 2 weasels, 4 guinea-pigs, and 4 rabbits with negative results Then from a dog, dying of a spontaneous "distemper-like" disease the same type of microorganism was grown from the purulent secretions of the eye and nose, from the lung, pericardial fluid, and liver A filtrate from the lung of this animal inoculated subcutaneously into a new dog led to death in 18 days after a severe attack of the respiratory type of distemper From this dog the same microorganism was again cultured from the lung, pleural exudate, and liver Inoculation of the 8th generation of this culture presumably produced distemper in 3 puppies The lungs, spleen, liver, and pericardial fluid of 9 healthy dogs were cultured in a similar manner but in no instance were these microorganisms encountered

In 1936, Shoetensack (88) reported that in 14 of 15 dogs which died of typical respiratory distemper, *A canis*, Type I, could be cultured from one or more organs—always from the lungs, and occasionally from the kidneys, brain, and "glands" *A canis*, Type II (biologically and serologically different from Type I) was found in only 3 of these dogs He believes that Type II is "seemingly unable to act as the first invader in the case of contagious respiratory type of canine distemper, and can be conceived to be acting only a part of the role in the disease, appearing together with the Type I organism in some cases of canine distemper of the contagious respiratory type Sometimes it is also found in spontaneous pneumonia in dogs" (87) Concerning prophylaxis with a "dead organism vaccine" prepared from pure cultures of *A canis*, I and II, Shoetensack states "The dogs which received two injections of 2 c c each of the vaccine have shown results which go to demonstrate the vaccine as a means of prophylaxis against infection from canine distemper" (88) The control dogs all died of severe distemper, and positive cultures of the Type I and II microorganisms were obtained from them The 5 vaccinated dogs lived longer, died of other conditions, and no Asterococci could be cultured from them.

A critical examination of the data leaves one in doubt as to the relationship between the virus of canine distemper and the microorganisms of the pleuropneumonia group which were so constantly present in the exudates and tissues of the sick dogs The existing data do not even permit a statement on the pathogenicity of the dog microorganisms, and further study of the entire question, including the possibility of a double etiology of canine distemper, certainly seems indicated In this respect some recent observations of Pinkerton (70) on mink distemper are of interest Finely minced lung, spleen, kidney, and bladder tissues from eight minks moribund or dying of distemper were spread over horse-serum-agar slants (Zinsser, Fitzpatrick, Wei medium) and incubated at 37° While the majority of the cultures remained sterile and no microorganisms or definite inclusions were found in films, several cultures representing lung, kidney, and bladder tissue from one mink and bladder tissue from another, developed irregular focal areas of clouding, 2 to 5 mm in diam-

eter, in the substance of the medium underlying certain of the tissue fragments Film preparations revealed a heavy growth of a minute, spiral microörganism which Pinkerton believed to have certain morphological characteristics in common with that of bovine pleuropneumonia This microorganism was carried for several transfers on the Z F W medium without tissue, and subinoculations on blood agar remained sterile Intracellular clusters of the organism were observed in the first culture on the Z F W medium, in which the cells were those of mink tissue, as well as in plasma-tissue cultures and in Maitland medium using guinea-pig lung and spleen It was then found that the microorganism would also grow in blood broth in which it produced a fine clouding After three transfers in this medium, large amounts of culture were inoculated into five ferrets known to be free of present or past infection with distemper, three subcutaneously and two intranasally All of them remained well for 6 weeks and subsequent inoculation with moderate doses of the original mink virus produced typical fatal distemper infection in all five of the ferrets I examined some films of this microorganism which Doctor Pinkerton was kind enough to send me, and my impression is that it cannot yet be included in the pleuropneumonia group chiefly because of the absence of the characteristic polymorphic forms and because not enough is known of its capacity to grow, or its type of growth, on solid media

Pleuropneumonia-like Microörganisms of Rats

"L_1" Since many rats normally carry *Streptobacillus moniliformis* in their nasopharynx (90), its symbiont or variant designated L_1 by Klieneberger, may be regarded as the first pleuropneumonia-like microörganism demonstrated in this species (44, 45) The peculiarities of this microorganism will be discussed separately later on, but it may be mentioned here that on at least one occasion L_1 has been isolated directly from the lung of a tame rat independently of *Streptobacillus moniliformis* (47)

"L_3" In 1937, Klieneberger and Steabben (46) examined the lungs of 19 rats exhibiting chronic "bronchopneumonia" or "bronchiectasis" and from 17 they isolated pleuropneumonia-

like microörganisms often in pure culture, which they at first called L_1 Subsequent immunologic and other studies (47) revealed however, that, with at least one exception, they were dealing with a distinct microörganism which was given the name L_3 Lungs of normal appearance from 10 rats were also cultured at that time but all with negative results In 1940, Klieneberger and Steabben (50) reported additional studies on the lungs of rats of different ages comprising a total of 251 laboratory rats and 17 wild ones Lung lesions were present in 108 laboratory rats but pleuropneumonia-like microörganisms were recovered from the lungs of 138, there were altogether 46 out of 139 rats, 8 months of age or younger, with lungs of normal appearance which yielded these microörganisms on culture The authors refer to all these microörganisms as L_3, although there is no indication that more than 4 strains were typed serologically There is still the possibility, therefore, that the pleuropneumonia-like microorganisms in the lungs of rats may belong to a variety of serological and biological types Among the 17 wild rats they found only one with lung lesions and that one yielded an L_3 microörganism confirmed by serological typing, the lungs of the remaining 16 gave negative cultures

Although it has not proved possible to produce any pulmonary lesions or any other pathogenic effects in rats with cultures of L_3 microörganisms, Klieneberger and Steabben (50) are inclined to regard them as having some connection with the causation of the natural disease Subcutaneous or intraperitoneal inoculation of L_3 cultures, particularly when mixed with agar, into mice has produced abscesses but there is as yet no evidence that the microorganism was multiplying in the mouse and not in the implanted agar, no symptoms, however, resulted from intracerebral inoculation in mice (50) Doctor Joel Warren informs me that intravenous inoculation of L_3 cultures in mice produced no apparent disease

"*L_4*" Early in 1938, Woglom and Warren (100, 101) reported that under certain conditions suspensions of *sarcoma 39* produced abscesses upon subcutaneous inoculation in rats These abscesses were transmissible by Berkefeld filtrates containing

no demonstrable bacteria Upon intravenous injection in rats the filtrable agent produced widespread suppuration involving especially the extremities, the testis, and the soft tissues about the head and larger joints The mouse was even more susceptible to this agent than the rat, but guinea-pigs and seemingly also rabbits were refractory, it could be cultivated in serial passage on the chorioallantoic membrane of the chick embryo Cytoplasmic inclusions were reported in the epithelial cells of the skin overlying the abscess

Somewhat later in 1938, Klieneberger (47) reported the isolation of a new pleuropneumonia-like microörganism from the "swollen submaxillary gland" of a rat She called it L_4 because it was culturally and serologically different from L_1 and L_3 Upon culturing some of the infective material of Woglom and Warren she discovered that the filtrable pyogenic agent was identical with the L_4 microörganism (48) The L_4 cultures could produce an abscess upon subcutaneous inoculation but had no effect whatever upon intravenous inoculation However, when some of the material from an abscess produced by a culture was injected intravenously, the effects of the original filtrable agent were reproduced Woglom and Warren (102) confirmed these findings In my hands, cultures of an L_4 strain given me by Doctor Warren produced "polyarthritis" and suppuration in the interscapular region upon intravenous inoculation in mice, none of the mice died and the "polyarthritis" disappeared spontaneously

Still another condition, in which the L_4 microorganism ultimately was shown to be of etiological relationship, came to light in 1938 Collier, (11, 12, 13, 16, 17, 18) working in Java, found that the spontaneous "polyarthritis" (swollen extremities) observed in a wild rat (*Rattus norvegicus*) could be transmitted to white rats in serial passage The infectious agent, which could not be identified with any cultivable bacteria, was shown to be widespread in the body and capable of producing a polyarthritis regardless of the route of inoculation Some of the rats died of a systemic infection, and in the survivors the arthritis cleared up spontaneously Findlay, *et al* (37) reported finding a similar

spontaneous "polyarthritis" among pure-bred laboratory rats in London They were able to transmit this condition to other rats with bacteria-free, gradocol-membrane filtrates and showed that the etiological agent is identical with a pleuropneumonia-like microorganism which they at first called "L_7" but which was subsequently found to be serologically identical with L_4 (49) This "L_7" strain was pathogenic for rats and mice but not for pigs, rhesus and cercopithecus monkeys, rabbits, guinea-pigs, voles and hedgehogs Later, Beeuwkes (1), working in Java in the same laboratory with Collier, reported the isolation of two strains, one from the infectious agent of spontaneous polyarthritis of the rat and the other from a similar agent obtained from rats after inoculation with material from a patient with acute rheumatic fever These two strains appeared to be identical biologically but since their serological type was not determined, one cannot, of course, regard them as representatives of L_4

Summary Three distinct biological and serological types have thus far been found in rats L_1, L_3, and L_4 L_1 occurs usually in association with *Streptobacillus moniliformis* and is not pathogenic without it L_3 has been found in normal lungs and in lungs with chronic bronchiectatic lesions, but it has not yet been found to be pathogenic in rats and the extent of its pathogenicity for mice has yet to be elucidated L_4 has been shown to be the cause of spontaneous polyarthritis of rats and apparently is also latent somewhere in the body, since it has also showed up in a "swollen gland," in transplantable tumors, etc L_4 is pathogenic for rats and mice Little is known of the natural habitat of these microorganisms in rats

Pleuropneumonia-like Microörganisms of Mice

These mircoörganisms were first encountered in mice in 1938 during the course of experiments with *Toxoplasma* (74, 75) and the virus of lymphocytic choriomeningitis (35), and the first strain was isolated simultaneously in America and England from the brains of mice infected with those agents During the next two years a number of other biologically and immunologically distinct species were found in mice and it was shown that their natural

habitat was in the conjunctiva, the mucosa of the nose and occasionally trachea, and oddly enough in the brain. Many different stocks of mice were shown to be carriers of these microorganisms (77, 80). Preliminary experiments have revealed that newborn mice do not harbor them, and acquire them only if their mothers or the other mice with which they are in contact are carriers. They make their first appearance in the nose, and the eyes have thus far not been found to be infected until after they have opened. Mice probably remain carriers during their entire lifetime. Thus far, there have not been encountered any spontaneous diseases in which any of these microorganisms are the etiological agents. And yet all of the known strains which appear to be harmless in their natural habitat (unless they are the cause of mouse catarrh) have proved to be highly pathogenic under experimental conditions in which they are brought into contact with the tissues for which they seem to have a special affinity. Work done with at least two types of this group has indicated that while *in vitro*, they can multiply in cell-free media, *in vivo* they grow only in association with or within the cells for which they have a special affinity. These microörganisms of mice vary sufficiently from one another as regards morphology, colony type, pathogenicity or tissue affinities, toxin production and antigenic make-up to permit their classification into a number of distinct species.

Type A (Sabin) and L_6 (Findlay, Klieneberger, MacCallum, and Mackenzie). While the Type A microörganism was first isolated from the brains of mice used in experiments with *Toxoplasma*, it has since been found on at least one occasion in the brain of a normal mouse, almost regularly in the eyes (conjunctiva) of carriers, in the nasal mucosa and in the lungs of mice which had received nasal instillations of various materials (77, 91). Regardless of what other type may be carried in the nasal mucosa, the microörganisms isolated from the eyes have, thus far, always been Type A. Upon intracerebral injection of brain or other tissues containing the Type A infectious agent, or of serum-broth cultures, there developed in most mice after an incubation period of 1 to 10 days, but usually on the 2nd or 3rd day, a very charac-

teristic turning or rolling on the long axis of the body with or without other nervous signs Some of the mice died, some continued with choreiform signs or hydrocephalus for months and years, while the majority recovered in a few days Two kinds of lesions were observed in the brain after intracerebral injection the first, consisting of destruction of the periventricular tissue followed by extensive infiltration with polymorphonuclear leucocytes, was present in all mice while the second consisting of almost complete necrosis and lysis of the posterior pole of the cerebellum was found only in mice which exhibited the turning or rolling signs Intracutaneous, subcutaneous, intramuscular, intratesticular, or intravenous injection as well as nasal instillation under ether anaesthesia, or administration by stomach tube of large amounts of infected mouse brain was without any obvious effect After intra-abdominal or intrathoracic injection, on the other hand, 20 to 40 per cent of the mice developed convulsions and the characteristic rolling within 17 to 48 hours and died shortly thereafter, revealing the same cerebellar change but not the periventricular destructive and inflammatory lesion found after intracerebral inoculation Extensive studies disclosed that after intra-abdominal and intrathoracic inoculation the infectious agent multiplied in the mesothelial cells of the peritoneum or pleura but was not present in the brain, suggesting that the lesions in the brain were caused by a toxin When this infectious agent was grown in glucose-serum broth, the cultures were not only as pathogenic as the infected tissues, but the presence of a true exotoxin, capable of reproducing the same nervous signs and cerebellar lesion after intravenous injection, was demonstrated in them (75)

Primary cultivation of the Type A microorganism in fluid media is facilitated by the addition of 0 5 per cent glucose About 48 hours after growth first becomes grossly apparent in such a medium, the culture is no longer pathogenic or viable This is associated with the development of acid, the pH dropping from about 7 8 to about 6 0 In the absence of added sugar this does not occur and subculture is possible for at least a month Morphologically the elements making up a fluid culture consist

chiefly of elementary bodies, minute rings or ovals with condensed bodies at one or both poles, and occasionally triangular or quadrangular structures in which the elementary bodies are linked by thinner bonds On solid media good growth is obtained only when the moisture is kept in by sealing the Petri dish The *fully developed colonies vary in size from 20 to 100* μ with only an occasional well-isolated one reaching 200 to 300 μ, and present central areas which are circumscribed, elevated, and darker than the rest Giemsa-stained impression films of such colonies present in addition to those found in fluid cultures much larger and more polymorphic structures When fluid cultures are injected intravenously into older mice which are not as susceptible to the toxin as younger ones, or when the microörganisms are injected after being separated from the toxin by centrifugation, approximately 20 per cent of the animals develop a polyarthritis which usually clears up in the course of a few weeks No cartilage destruction or ankylosis has been observed with this type During the course of the arthritis an occasional mouse develops choreiform signs The pathogenicity of this strain has remained even after more than 100 subcultures *in vitro* Rabbits, guinea-pigs, and rhesus monkeys have not been found susceptible

The microorganism isolated by Findlay, *et al* (35) from mice with "rolling disease" and called L_5 was reported by them to be immunologically related to or identical with the Type A microorganism on the basis of cross-immunity tests in mice However, there are some significant differences in pathogenicity between the two Neither their original infectious agent in the mouse brains, which had a lower titre, nor the L_5 cultures were pathogenic after intraperitoneal or intrathoracic injection Not only were nervous signs absent but there also appeared to be no local multiplication since it could not be recovered from the liver or spleen 3 days after inoculation Furthermore the L_5 culture produced no apparent signs of disease after intracerebral injection unless it was mixed with agar or virus-infected mouse brain No cerebellar lesion was reported and there is as yet no evidence that L_5 produces the neurotoxin It would, therefore, be inadvisable to call the L_5 and Type A microörganisms identical until more work has been done,

although Doctor Homer Swift informs me that the two are serologically identical

In 1939, Sullivan and Dienes (91) produced pneumonia in mice by serial passage of mouse-lung suspensions by means of *nasal instillation under ether anaesthesia From the lungs of* such mice they isolated several strains of pleuropneumonia-like microörganisms They stated that "when injected intravenously, intraperitoneally or subcutaneously in the two strains of mice at our disposal, our cultures failed to produce any clinical-pathological phenomena" Sullivan and Dienes were kind enough to send me two of their strains which I grew in 10 per cent rabbit-serum broth or 30 per cent ascitic-fluid broth with 0 5 per cent glucose added in both instances After several passages growth appeared in 24 hours, and intravenous injection of 0 5 ml amounts of 24 or 48 hour cultures into 3- to 4-week-old mice produced within 1 to 2 hours the typical convulsions and rolling characteristic of the neurotoxin effect, with the majority of animals dying in 3 to 4 hours This toxic effect was completely neutralized by the Type A antiserum, and results of agglutination tests showed conclusively that the two strains of Sullivan and Dienes were identical with the Type A microörganism *While these strains were indeed isolated from lungs with* pneumonia, these authors presented no evidence that they were the cause of the pneumonia or that the cultures could either produce pneumonia or immunize against it The relationship between the experimentally produced pneumonia in mice and the Type A microorganisms is, therefore, still to be investigated

The Type B Microörganism This microorganism was first cultured from the brain of a normal mouse (75, 76) and has subsequently been found in the nasal mucosa (77, 80) Morphologically it differs from the Type A microörganism in that it has more complex and polymorphic structures in fluid media Especially noticeable on dark-field examination are the long undulating and vibrating thin filaments which are attached to the ring structures and which are usually first seen during the second day of grossly apparent growth The Type B colony differs from that of Type A in not having the central, raised, dark,

nipple-like structure but rather what seems to be a meshwork of vacuoles. Acid is produced in the presence of glucose and the fluid culture loses both its pathogenicity and viability 2 to 3 days after growth becomes grossly apparent. It is further differentiated from Type A by not producing a toxin, by its great affinity for the joints and inability to multiply in most other tissues, and by being immunologically distinct. After intracerebral injection of a culture at the height of growth, the mice remain well and there is not even multiplication of the microörganism. Several rapid brain-to-brain passages lead to the complete disappearance of the microorganism rather than to adaptation and increased virulence. Intracutaneous, subcutaneous, intramuscular or intrathoracic injection or nasal instillation with or without ether anaesthesia induced neither arthritis nor any other local or systemic disease. Arthritis was produced in practically 100 per cent of mice, however, when 0.5 ml. of a 24- or 48-hour culture was injected intravenously and somewhat less often when 1 ml. was injected intra-abdominally. The microorganisms disappear from the peritoneal cavity 24 hours after intra-abdominal injection and there is no multiplication in the viscera after intravenous injection. Swelling of the joints may appear as early as 4 to 5 days, and then the arthritis is migratory, new joints becoming involved as others recede. Fusiform swellings of isolated digits occur frequently. The process is progressive and chronic leading often to ankylosis, especially of the knees, "wrists" and "ankles", after 2 to 5 months. Although it has not yet proved possible to obtain positive cultures from the joints before or during the first day or two of clinically apparent arthritis, there is no difficulty in securing growth in fluid or solid media somewhat later and positive cultures have been gotten as late as 10 weeks after intravenous inoculation. The affected animals appear otherwise healthy. Pathological changes are limited to the joints and consist chiefly of proliferation in the synovial membrane, the capsule, the perichondrium, and necrosis of the articulating cartilage. Cultures have retained their pathogenicity even after 50 to 60 passages *in vitro* but there is an indication that it is diminished after prolonged cultivation *in*

vitro Inoculation of large amounts of virulent culture into rabbits and guinea-pigs has been without effect

Types C, D, E and Other Pleuropneumonia-like Microorganisms Reported in Mice Several strains of the Type C microörganism have been isolated from the lungs of mice which had previously received various materials by nasal instillation under ether anaesthesia (77) Type C produces a progressive polyarthritis in mice following intravenous injection, but differs from Type B in its antigenic make-up and probably also in its capacity to multiply in other tissues in addition to those of the joints Type C produces no neurotoxin and is also immunologically distinct

Four strains of Type D were encountered in the nose, lung, and brain of mice (80) It is immunologically distinct from the other types, has no neurotoxin, and produces progressive arthritis in mice after intravenous injection The same is true of a single strain isolated from the nasal mucosa of a mouse and called Type E because it differed in its antigenic make-up from all the others Not enough work has yet been done to permit a statement on the tissue affinities of these types

Findlay, *et al* (36) inoculated intracerebrally into mice the blood of splenectomized mice containing *Eperythrozoon coccoides* From the brains of the inoculated mice they isolated on 6 occasions a microorganism which they called L_6 It is not identical with *E coccoides* and is serologically distinct from L_1, L_3, L_4, and L_5 Its colony has a coarse, globular surface and in liquid medium it forms little clumps which are composed of fairly large globules (49) It is stated (38) that L_6 produces a fatal encephalitis upon intracerebral injection in mice and "arthritic changes" at the site of inoculation in the pad of the foot in 30 to 50 per cent of animals Klieneberger (49) also reports the isolation by Dr H Jahn, from the swollen joint of a mouse, previously inoculated with *Streptobacillus moniliformis*, of a pleuropneumonia-like microörganism which is not L_1, and is called "M55" It is reported to cause arthritis but has not been typed, and its relation to the other members of this group is unknown

Edward (31) isolated a number of strains of pleuropneumonia-like microörganisms from the pneumonic lesions of mice which

had been given nasal instillations under ether anaesthesia of mouse lung suspensions These strains did not form distinct colonies on solid medium, but since it is not stated whether or not the evaporation of moisture was prevented, the lack of colonial development might have been due solely to the conditions of cultivation Fluid cultures, however, presented morphological forms similar to those of other members of the group ("granules, ring forms of various sizes, forms showing filamentous budding, and thin bacillary bodies with several rounded swellings along their length") including reproductive units which passed gradocol membranes with an A P D of 0 45 μ "All the strains were tested for their ability to ferment glucose, fructose, galactose, xylose, arabinose, rhamnose, maltose, sucrose, lactose, raffinose, dextrin, inulin, salicin, mannitol, dulcitol and glycerol, but production of acid from any of these could not be definitely established A temperature of 55°C for 15 minutes killed the organisms They resisted 45° and 50°C for 15 minutes but not for 30 minutes" With respect to sugar fermentation as well as heat resistance, Edward's strains would appear to differ from the Type A and Type B microorganisms, since the latter definitely produce acid from glucose and do not resist 45° and 50° C for 15 minutes They are also different in their pathogenic properties in that intra-abdominal or intravenous injection of cultures of strains 1, 2, and 4 (all of one serological type) produced neither arthritis nor any nervous signs suggestive of the presence of a neurotoxin This is in agreement with the fact that strain 4 was not agglutinated by either the Type A or Type B antiserum, but was agglutinated to 1 160 by the Type C antiserum (Personal communication from Doctor Edward) Since the Type C serum had a titre of at least 1 1000 for the homologous microorganism, one cannot regard those strains (i e 1, 2, 4, 5, and 6, all of one serological type) as being identical with Type C Strains 3 and 7 were serologically distinct from the others and Doctor Edward informed me that Type A antiserum agglutinated strain 3 to 1 320 which only suggests antigenic relationship since this serum agglutinated the homologous microorganism to a dilution of 1 6000 Strain 3 was agglutinated to 1 40 by the Type C serum

and not at all by the Type B serum Although the relationship of these strains to Types D and E is unknown, their failure to produce arthritis would constitute at least one point of difference Although Edward expressed the belief that the microörganisms he isolated from the lung lesions were actually the cause of the pneumonia, it is to be noted that only the early subcultures could produce pneumonia and that no tests were made to rule out (a) the presence of a virus, or (b) to show cross-immunity between the microörganisms and the pneumonia-producing agent Horsfall and Hahn (42) were able to show that while pleuropneumonia-like microörganisms were present in the lungs of mice with a spontaneous virus pneumonia, they were not identical with the etiological agent of the pneumonia

From this review it is apparent that mice are carriers of a large variety of immunologically and biologically distinct microorganisms of the pleuropneumonia group and also that all the strains which have been tested are different from those that are known in the rat It is of interest to note, therefore, that in at least two instances mice which had been quartered with rats in the same room or building were found to be carriers of rat microörganisms In 1939, Dr Joel Warren isolated an L_4 microörganism from the brains of mice used for passage of herpes virus by the intracerebral route, and Findlay, *et al* (37) of an L_3 microörganism from the brain of an apparently healthy mouse

Pleuropneumonia-like Microörganisms of Guinea-pigs

The existence of an independent member of the pleuropneumonia group has not yet been demonstrated in guinea-pigs, but that such may be present in association with some of the bacteria is suggested by the work of Klieneberger In 1935, she reported the isolation on two occasions of a gram-positive streptococcus or streptobacillus from the nasopharynx of healthy guinea-pigs Associated with the bacterial colonies were "minute" colonies which she called L_2, but these were never isolated and their essential nature remains obscure (44) In 1940 she described the isolation of a culture resembling *Streptobacillus moniliformis* from large cervical abscesses of guinea-pigs Since *S m*

formis of rats is not pathogenic for guinea-pigs, it could not be dismissed as merely a cross-infection From the guinea-pig strain, she obtained a microörganism, resembling L_1, but these cultures were lost with the outbreak of war before any serological work was done with them (49)

Pleuropneumonia-like Microörganisms of Man

In 1937, Gerlach (40) was probably the first to suggest the presence of filtrable microörganisms of the pleuropneumonia group in man His illustrations and protocols, however, present convincing evidence to the contrary and suggest that he misinterpreted precipitation in the media In the same year Dienes and Edsall (22) reported the isolation of a pure culture of a pleuropneumonia-like microörganism from a suppurating Bartholin's gland of a laboratory worker At that time, however, they were inclined to attribute the infection to the patient's contact with rats While the strain was lost before its biological and serological identity could be established (there was never any reason for calling it L_3), the more recent work of Dienes (28) suggests that contact with rats might have had nothing to do with the infection Following the demonstration of the special pathogenic properties of the Types A and B mouse microorganisms, Swift and Brown (93) reported the isolation of pleuropneumonia-like microörganisms from patients with rheumatic fever, but they subsequently pointed out that their direct "cultures" were misinterpreted while the ones obtained after passage through mice were biologically and immunologically identical with those normally carried by these animals (77) Many attempts by a number of investigators to cultivate such microörganisms from exudates and tissues of patients with rheumatic fever or rheumatoid arthritis have all been unsuccessful (38, 77, 81, 91)

In view of the fact that mice were shown to carry these microorganisms in their eyes (conjunctiva) and nose, swab cultures from these regions obtained from 100 human beings were studied by Sabin and Johnson (81), but without success They observed,

however, that when human tonsils were minced and smeared over 30 per cent ascitic-fluid-agar plates, minute colonies (20 to 40 μ in size) resembling those of the pleuropneumonia group sometimes grew out in between the larger bacterial species These colonies, which they called "X" colonies, occurred in 3 out of 60 cases in which children's tonsils were studied, and on 2 occasions in another group of 103 children The "X" colonies, however, could not be passaged either *per se*, or in the presence of the associated bacteria or rich filtered extracts of tonsillar tissue, and no studies which would permit a decision concerning their relationship to the pleuropneumonia group were, therefore, possible In this connection, it is of interest to quote the following sentence from a paper by Seiffert (85) dealing with the saprophytic filtrable microörganisms in soil, manure, etc "Es ist noch zu erwahnen dass ich ahnliche Mikroorganismen einmal aus Speichel und dreimal aus Sputum von Bronchiektatikern gewinnen, aber nicht in Passage weiterzuchten konnte" Whether or not there exist in the respiratory tract of human beings microorganisms of the pleuropneumonia group with special requirements for *in vitro* cultivation is a question that is raised by these observations and may well repay further investigation

A recent brief report by Dienes (28) brings the first evidence that is more than suggestive that such microörganisms exist in human beings Cultures from the genital tract of female patients who had gonorrheal pelvic infections were studied on an ascitic-fluid-agar medium, and pleuropneumonia-like colonies were encountered in about one-third of the cases After 48 hours these colonies were often only 10 to 20 μ in diameter, but they developed to considerably larger size on passage Four strains were thus isolated in pure culture Since I disagree with Doctor Dienes on some of his morphological interpretations, I was frankly skeptical, until he was good enough to send me not only preparations of these colonies but also a Giemsa-stained film of the growth in a fluid culture I found these to be morphologically absolutely typical of the pleuropneumonia group and indistinguishable from the forms observed in the cultures of the mouse

microorganisms Doctor Dienes informs me that his strains grow and survive in broth cultures but the growth is very scanty Further studies in this new direction are of the greatest interest

Pleuropneumonia-like Microörganisms as Saprophytes in Sewage and Other Decomposing Matter

In 1936, Laidlaw and Elford (52) reported a new group of filtrable microörganisms, obtained from raw sewage originating in four London districts, but not detected in London tap water, nor in fecal material from man, pig, rabbit, or rat Sewage was filtered through gradocol membranes with an A P D of 1 0 μ, 0 8 μ, or 0 6 μ and seeded into Fildes's broth or agar Growth occurred at 22° and at 37° but was optimum at 30° In broth cultures they found granules, varying in size from small cocci about 0 5 μ in size to small dots which were imperfectly resolved, rings, single or paired, with an occasional one showing a thickening or granule at the periphery or a short filament attached to the margin The most turbid cultures contained as many as 3 to 10 billion reproductive units per ml Typical pleuropneumonia-like colonies, i e minute, round, granular colonies with well-marked dark centers and lighter peripheral zones, developed on the solid media The various strains fell into three serological types which they called A, B, and C Pathogenicity was tested by feeding cultures to rats, by intravenous inoculation in rabbits, inhalation, subcutaneous and intra-abdominal inoculation in mice, all with negative results Cultures from the peritoneal cavity of inoculated mice were sterile within 24 hours

Seiffert (84, 85) confirmed these results by showing that similar filtrable microörganisms were present in Germany in soil, compost, decomposing leaves, and manure At least two of the German strains are serologically identical with and the others are all related to those isolated in England (49) In addition, Seiffert (84) pointed out that "Diese mehrfach wiederholten Versuche durften dafur sprechen, dass es in der freien Natur neben filtrablen, an der Grenze der Sichtbarkeit stehenden Mikroorganismen wahrscheinlich auch solche gibt, die nur durch irgendwelche Lebensreaktionen nachweisbar sind " From compost which had

been filtered through membranes of smaller porosity he obtained a *transmissible*, enzyme-like effect, i e decomposition of starch, which could be carried on for 5 to 8 passages There was no decomposition of starch in corresponding dilution experiments, nor after the addition of chloroform There was no definite turbidity in the cultures and no visible growth on solid media Liquefaction of gelatin could also be obtained for 6 passages

L_1 AND STREPTOBACILLUS MONILIFORMIS QUESTION OF SYMBIOSIS OR VARIATION AND ITS SIGNIFICANCE FOR THE MICROORGANISMS OF THE PLEUROPNEUMONIA GROUP

Streptobacillus moniliformis (54), a gram-negative pleomorphic bacillus, is a normal inhabitant of the nasopharynx of rats (90) While its pathogenicity for rats is doubtful or nonexistent, it produces a highly fatal generalized pyogenic infection in mice (55, 56), which because of the localization of the process, that is essentially one of multiple abscess formation, in the heart muscle, joints, and elsewhere has called forth erroneous comparisons with rheumatic fever (55) In human beings, however, *S moniliformis* does give rise to diseases (rat-bite fever and Haverhill fever) clinically reminiscent of certain manifestations of rheumatic fever, i e recurrent attacks of fever, erythema, and polyarthritis (21, 34, 54, 68)

From the work reported by Klieneberger and others it became apparent that all strains of *S moniliformis* were associated with or gave rise to another microörganism, called L_1, which possessed many properties in common with the microorganisms of pleuropneumonia and agalactia Separation of the two microorganisms by the usual bacteriological methods failed and it was not until Klieneberger (45) found that the two were not equally resistant to ageing or heat that such separation was possible When a 4- to 8-day-old broth culture kept at 37°, or a fresh culture heated at about 53° for 5 to 15 minutes was seeded on 10 per cent horse-serum-agar plates, there developed after a few days' incubation 5 to 300 of the usual, large (3–5 mm), mixed *S moniliformis* colonies and in between them a number of minute colonies which could be detected with a magnifier or a micro-

scope When fully developed after about 4 days' incubation the smaller colonies were about one-fifth the size of the larger mixed variety and were characterized by a dark, granular centre and a clearer ring zone Subcultures of the small L_1 colonies on the same medium failed, however, and it was not until Klieneberger used a "special medium" [heart-muscle-infusion-peptone broth (pH 7 6–8 0) with boiled blood (5 per cent defibrinated blood), 20 per cent of horse serum, 20 per cent of a sterile filtrate of a *S moniliformis* serum-broth culture, and 2 per cent agar] that isolation and continued maintenance of L_1 in "pure" culture was possible After many subcultures old strains of L_1 were able to grow on 30 per cent horse-serum agar without the added filtrate When it is grown in fluid medium the broth itself remains clear and large colony clumps are found sticking to the wall or as a sediment at the bottom of the tube Klieneberger has stressed the necessity of repeated purifications of L_1 cultures, since some strains were found often to revert to *S moniliformis* in the early passages She has maintained several strains of L_1 cultures for over 100 to 300 passages without reversion to *S moniliformis* either in her own hands or in anybody else's (personal communication from Doctors L Dienes and J Warren) Because of these facts and because a pleuropneumonia-like micoörganism, morphologically, culturally, and immunologically identical with L_1, was isolated on at least one occasion from the lungs of a rat without *S moniliformis*, Klieneberger has maintained that the *Streptobacillus moniliformis* represents an intimate symbiosis between the streptobacillus and L_1 This concept was challenged by Dienes (23, 24) who suggested that the L_1 microörganism was but a variant of the streptobacillus chiefly on the grounds that the strains of L_1 which he isolated and which apparently grew in pure culture on solid medium reverted to *S moniliformis* in broth cultures, and partly because, in his opinion, the morphology of the L_1 microörganism as well as of other pleuropneumonia-like microorganisms does not differ materially from that of bacteria Klieneberger's (49) recent comment on this controversy is very enlightening

"To make the position quite clear it should be pointed out that Dienes and the writer have both been able to separate the so-called L_1 form from the parent culture of *S moniliformis* and have maintained it in pure condition They both agree that the L_1 consists of granules, filamentous forms and pleomorphic bodies, while the *S moniliformis* cultures contain bacillary chains in addition to these elements The two chief points of disagreement are the following Dienes finds his L_1 strains reverting into the parent culture under certain conditions, while the writer's strains [*i e the highly purified ones—A B S*] have so far not reverted under similar conditions Dienes believes further that the globular forms are swollen bacilli and that the filamentous forms in the L_1 correspond to the bacilli, while the writer believes that the globular forms develop independently and that they resemble as well as the filamentous and granular forms elements seen in the cultures of pleuropneumonia and agalactia

"The reasons why the writer is still in favor of the symbiosis hypothesis are the following The bacillary elements of *S moniliformis* show by their shape, arrangement, multiplication mode and their bacterial "rigidity" that they are true bacilli while the L_1 growth contains no ordinary bacillary forms, but elements resembling those of the organism of pleuropneumonia bovis Like pleuropneumonia the L_1 cultures contain a large amount of small granules of different shape some of which are not much larger than vaccinia bodies, the globules which form an integral part of both are not to be compared with swollen bacterial forms as Dienes suggests Besides possessing a similar morphology, L_1 and the organisms of the pleuropneumonia group show also the same colony type There is further the regular filterability of L_1 and the pleuropneumonia-like organisms through some of the coarser filters such as the Berkefeld V candle It seems a reasonable conclusion from these data that the L_1 is itself a pleuropneumonia-like organism and consequently unlikely to prove a variant growth form of the streptobacillus There is the further argument that if the L_1 is a variant form of *S moniliformis*, the now numerous strains of the L series occurring independently should be derived from streptobacillary mother strains, but evidence of the existence of such is not forthcoming"

One of the cardinal characteristics of the members of the pleuropneumonia group is the possession of reproductive units which are in the range of 125 to 250 mμ in size Filtration

through Berkfeld candles, which often permit ordinary bacteria to pass and whose pores are large and irregular, cannot be relied upon for this essential information It is regrettable, therefore, that no filtration experiments with the L_1 cultures through gradocol membranes of known A P D have as yet been carried out or reported If L_1 cultures could be proved to contain such minute reproductive units, and if these minute units, isolated from early passage cultures by gradocol membrane filtration, were shown to be capable of reproducing *S moniliformis*, the case for the variant hypothesis would be definitely stronger Since the bacillary elements in a culture of *S moniliformis* incubated for 2 days or longer undergo rapid disintegration, it would also be significant to determine whether or not minute elements of the same order of magnitude which could reproduce the original streptobacilli could be obtained by gradocol filtration Since *S moniliformis* is a "mixed" culture it is not surprising that a serum prepared against it agglutinates the L_1 microörganisms as well as or better than a homologous serum, and *vice versa* The preliminary results with agglutinin-absorption reported by Dawson and Hobby (21) do not warrant the conclusion that *S moniliformis* and L_1 have an identical antigenic make-up It is perhaps of interest in this respect, that Klieneberger (49) found that immune serums against the L_4, L_5, L_6, and *Asterococcus canis* I microorganisms agglutinated L_1 suspensions in titres varying from 1 10 to 1 80 while the immune serums against other types as well as various bacterial immune serums and normal serums had no effect on these suspensions There is a suggestion, therefore, that L_1 is not in a class by itself but rather that it may possess a common antigen with some of the other members of the pleuropneumonia group

To what extent, if any, L_1 contributes to the pathogenicity of *S moniliformis* is obscure since, in pure culture, it is completely nonpathogenic It would, therefore, help to elucidate further the relationship between these microörganisms if it were known whether or not immunization with L_1 produced resistance in mice against infection with *S moniliformis*

Knowledge of the true relationship between L_1 and *S monili-*

formis would greatly add to our understanding of the filtrable microorganisms of the pleuropneumonia group For if L_1 is proved beyond doubt (proper filtration experiments still need to be carried out) to belong to this group, its origin as a variant from *S moniliformis*, if proved, would be an indication of how the other members of this group might have originated at one time On the other hand, if it is proved to be a symbiont, the failure, thus far, to find any strain of the streptobacillus, which is free from L_1 or which can be experimentally separated from it, suggests an association by far more intimate than that represented by ordinary symbiosis The symbiosis of L_1 with *Clostridium tetani* and *C. tetanomorphum* which Klieneberger (47) was able to establish experimentally and to maintain in over 100 passages, differed from that of *S moniliformis* in that both microörganisms could be recovered in a pure state Klieneberger has also encountered a gram-positive streptococcus or streptobacillus in the nasopharynx of guinea-pigs, a microörganism grown from an extracted human tooth (44), and gram-positive cocci from the skin of a pig (49) inoculated with swine-pox which gave every appearance of existing in "symbiosis" with microörganisms similar to L_1, but she was unable to separate the L_1-like elements in pure culture With regard to the peculiar large forms which have on occasion been observed in *Bacterium fundiliforme* (syn *Fusobacterium nucleatum*), *Hemophilus influenzae* and a *Flavobacterium* (26, 27, 49), Klieneberger (49) remarks that "the mere observation of swollen bodies or globular forms is not sufficient evidence to prove the existence of a filterable, pleuropneumonia-like symbiont or growth phase in these cultures"

FILTRABILITY AND SIZE OF MINIMAL REPRODUCTIVE UNITS

The development of reproductive units or elementary bodies, of the same order of magnitude as that of the larger viruses, in their life cycle constitutes one of the cardinal characteristics of the members of the pleuropneumonia group Filtration through the ordinary bacteriological filters cannot be relied upon to establish this important property, and only data obtained by filtering suitably prepared material through gradocol membranes

can be used in estimating the size of the minimal reproductive unit Laidlaw and Elford (52), in writing of the sewage micro-organisms, stated "In its general filtration behavior the organism resembles bovine pleuropneumonia and agalactia and contrasts with viruses and bacteriophages, which, individually, have been found to be relatively uniform in particle size as evidenced by the fact that no appreciable drop in filtrate concentration, as compared with the original, is detected until the porosity of the membrane used is about twice the true end-point value" This marked drop in titre following filtration through membranes of relatively large A P D was also found by Edward (31) in tests on one of the strains he isolated from mouse lungs The smallest units or elementary bodies actually form only a very small proportion of the total number of reproductive units that are present in a culture

The larger polymorphic structures in the cultures may be a source of error in filtration experiments and should be removed by centrifugation and preliminary filtration through membranes with average pore diameters of 0 7 to 0 9 μ As an example may be mentioned the experience with the Type A microörganism of mice With the infectious agent in the form of mouse-brain suspension in broth which had been highly centrifuged and well diluted, it passed a 980 mμ membrane but was retained by membranes with an A P D of 848 mμ or smaller When the 980 mμ membrane filtrate was used for further filtration, it passed a 720 mμ but was retained by 628 mμ When a culture of the Type A microörganism in serum broth which exhibits only a diffuse slight opalescence was filtered without preliminary centrifugation it passed a 584 mμ membrane but was retained by the membranes with an A P D of 500 mμ or smaller, after preliminary, high-speed, horizontal centrifugation of the culture it passed the 500 mμ membrane but was retained by that with an A P D of 396 mμ On this basis one might estimate the size of the minimal reproductive unit of the Type A microörganism at 200–300 mμ, but further tests need to be carried out with cultures that have been better prepared Thus the same filtration end-point was obtained with a centrifuged culture of the Type B

microörganism, but when the 584 mμ membrane filtrate was used for further filtration it was found that even the 322 mμ membrane filtrate yielded positive cultures The filtration end-points reported by Findlay, *et al* (35) for L_5 both in tissue suspension and in culture are practically identical with those for Type A given above, except that it would appear that they probably did not centrifuge their culture before filtration Regarding the filtrability of L_7 (the etiological agent of polyarthritis in the rat and serologically identical with L_4) Findlay, *et al* (37) reported that it passed membranes with an A P D of 560 and 440 mμ but not that of 300 mμ Since they did not indicate how the culture was treated preliminary to filtration, the final estimate of size must await a more detailed report

TABLE 1

Data on the size of the minimal reproductive units

MICROÖRGANISM FROM	A.P D OF MEMBRANE PERMITTING PASSAGE	A.P.D OF MEMBRANE WITH COMPLETE RETENTION	ESTIMATED SIZE OF MINIMAL REPRODUCTIVE UNIT
	mμ	*mμ*	*mμ*
Pleuropneumonia bovis	250–300	250	125–175
Agalactia	?	300	150–225
Sewage	260	250	125–175
Mouse lung (Edward)	450	330	165–247

The pleuropneumonia, agalactia, sewage, and one of Edward's mouse-lung microorganisms are the only ones on which properly performed filtration experiments have been reported, although additional tests would be desirable on the mouse organism because the gap between A P D 330 mμ and 450 mμ is somewhat too large Table 1 presents some of the essential data

TOXIGENICITY AND IMMUNOLOGICAL REACTIONS

Toxin Production

The first suggestion that a microörganism of the pleuropneumonia group may produce an exotoxin came when Nocard, *et al* (63) observed that the presence of collodion sacs, containing

growing cultures of pleuropneumonia bovis, in the abdominal cavity of rabbits often led to extreme cachexia and death Since there were no obvious lesions at necropsy and the microorganisms could be cultured neither from the blood nor from the viscera, and since the implantation of many collodion sacs containing only uninoculated medium was not harmful to the rabbits, they concluded that they were dealing in all probability with the diffusion from the collodion sacs of a toxin elaborated by the growing microörganisms They stated, in fact "Voilà donc un nouvel exemple d'un animal très sensible aux toxines d'un microbe contre lequel il est pourtant tout à fait réfractaire " In keeping with this is the observation that cows may die after subcutaneous injection of infected lymph or culture, although the only lesion is at the site of inoculation It is surprising, therefore, that no work appears to have been done or reported on attempts to establish whether or not the microorganism of pleuropneumonia bovis actually gives rise to a true exotoxin

There is, however, one member of the pleuropneumonia group, i e the Type A microörganism of mice, which unquestionably produces a true exotoxin during its growth both *in vivo* and *in vitro* (74, 75) It is called a true exotoxin first because it is present in sterile filtrates during the early period of growth (i e even before there is gross evidence of growth), second because it is thermolabile being destroyed at 50° in 30 minutes but not at 45° in the same period (the microörganism itself is killed at 45° in 15 minutes), and third because it is antigenic being specifically neutralized by antitoxin The existence of this toxin was suspected even before its isolation from cultures, because the extensive brain lesions, which were found in mice dying 17 to 48 hours after intra-abdominal or intrathoracic injection of the infectious agent, were not associated with the presence of any transmissible agent The production of this toxin *in vivo* is of special interest because all the indications are that under those circumstances the Type A microörganism multiplied only in the mesothelial cells of the peritoneum and pleura There is thus an example of a toxin elaborated in the cells of one organ producing its effect by acting on those of another (the brain) The evidence

that the toxin produced *in vivo* is identical with that *in vitro* is based on the fact that they both give rise to the same clinical signs, produce the same neurolytic change in the posterior pole of the cerebellum, and are immunologically identical

The amount of toxin in a culture is relatively small, an intravenous injection of 0 1 to 0 5 ml being required to demonstrate it It disappears from the cultures after about 2 days of growth Three-weeks-old mice are most susceptible and animals older than 2 months usually do not react After intravenous injection of the whole culture or a Seitz filtrate the first nervous signs, i e , convulsions or rolling, may appear as early as 5 to 10 minutes, usually within 1 to 2 hours, and in older mice may be delayed for 6 to 9 hours Most mice die within a few hours or less after inoculation, these show no lesions in the brain or anywhere else The mice that survive with persistent nervous signs, show the typical neurolytic lesion in the cerebellum as early as 18 hours after inoculation The toxin continues to be produced in cultures even after more than 100 subcultures *in vitro* In preliminary tests on rats carried out together with Doctor Joel Warren it was found that intravenous injection of 1 ml of an active "A" culture produced nervous signs and death within a few hours in four 36-day-old animals but was harmless in adult rats

Similar tests in mice with cultures of all the other pleuropneumonia-like microorganisms of mice, of L_3 and L_4 of rats, and of an old passage strain from pleuropneumonia bovis yielded no evidence of the presence of a toxin

Immunological Reactions, Groups, and Types

Immunization of rabbits with cultures of the microörganisms of the pleuropneumonia group leads to the development of agglutinins, complement-fixing and neutralizing antibodies The development of antibodies during the course of actual infection in susceptible hosts seems less definite, although some animals with pleuropneumonia or agalactia have agglutinins Also no neutralizing antibodies were found in the serum of recovered animals that were resistant to reinfection with the neurolytic, Type A microörganism of mice (74), the filtrable pyogenic agent

(L_4) of rats (101), and the microorganism of rat polyarthritis (13, 18) Neutralizing or protective antibodies, however, are easily demonstrable in the sera of immunized rabbits (79) and in hyperimmune sera prepared in sheep, goats, and horses (6, 30, 64)

The members of the pleuropneumonia group have been differentiated into a large number of distinct serological types with the aid of the agglutination reaction All the strains from pleuropneumonia bovis appear to belong to a single serological type (47, 94) which is not related antigenically to any other member of the group The same is true of all the strains from agalactia Those found in the dog belong to two distinct serological types which while not related with one another may according to Klieneberger (49) have a common antigen with some of the microorganisms of rats and mice Three distinct serological types have thus far been identified among the microörganisms isolated from rats, and five distinct types among those isolated from mice The existing data indicate that many of the murine strains have one or more antigens in common, some being more closely related than others The microörganisms originally isolated from sewage were found to belong to three serological types A, B, and C, with A and B possessing an antigenic relationship Klieneberger (49) compared all the available saprophytic microörganisms from England (Laidlaw and Elford) and Germany (Seiffert) with one another and with the parasitic ones and found (a) that they all fell into the three original types with Type C consisting of a single strain unrelated to any other member of the group, and (b) that the saprophytic and parasitic microorganisms had no antigenic relationship, excepting that the L_4 serum agglutinated many of the saprophytic strains to a titre of 1 10 or 1 20, although none of the saprophytic sera agglutinated L_4

It is interesting to note (a) that each microörganism that has distinguishing characteristics as regards biological and pathogenic properties is also serologically distinct, and (b) that each animal species seems to carry its own serological types, with the possible exception of some of the rat strains which on rare occasions may be transmitted to mice under natural conditions

PATHOLOGY

There is probably no pathological picture that is characteristic of effects of the pathogenic members of the pleuropneumonia group Some of them seem to have distinct tissue affinities while others have not Thus the microörganism of agalactia, regardless of the route of inoculation, can localize in the joints, the eyes, and the mammary glands of the lactating animals, the Type B microörganism of mice, after intravenous or intraperitoneal injection, will localize and multiply only in the joints, the Type A microörganism of mice will multiply in the cells of the peritoneal or pleural coverings of the organs but not in their parenchymal cells On the other hand, there are others that will multiply at the port of entry without spreading very far Thus, when infected lymph or a virulent culture from pleuropneumonia bovis is injected subcutaneously it produces a severe local reaction but not pleuropneumonia, the same is also true, to a certain extent, of the L_4 microorganism of the rat which will produce abscesses at the site of injection and will multiply wherever it is distributed after intravenous injection

The question of whether or not cytoplasmic or intranuclear inclusions occur is one which requires further study Meyer (58), Gaiger and Davis (39) found no such inclusions in bovine pleuropneumonia, and Tang, *et al* (95) could find none in the cells of the chorioallantoic membrane of the chick embryo inoculated with the pleuropneumonia microorganism Woglom and Warren (101) reported the presence of acidophilic, cytoplasmic inclusions (readily seen in their photographs) in the epithelium overlying the abscesses of 12 to 24 hours' duration which were produced by the filtrable pyogenic agent subsequently shown to be identical with L_4 These inclusions have not yet been reproduced with cultures, but further study of their relationship to the microörganism is definitely indicated I found the Type A microörganism of mice scattered throughout the cytoplasm of the mesothelial cells of the peritoneum and pleura when Giemsa-stained films of fresh tissue were examined, in sections of fixed tissues, however, I found more compact inclusions in the cyto-

plasm of the pleural cells, but was unable to see them in the peritoneal cells over the liver and spleen Sections of the joints of mice with experimental arthritis, produced by either type Type A or Type B, showed neither inclusions nor any other intracellular or extracellular structures which could be identified as the microorganisms

The primary attack of the microorganism of bovine pleuropneumonia appears to be on the connective tissue with the development of marked edema and large amounts of serous exudate In the natural disease it is the interlobular and subpleural connective tissue network that is affected with subsequent vascular thrombosis and pulmonary necrosis There may be only a fibrinous pleurisy over the consolidated lung and no effusion in the pleural sac but with large collections of fluid underneath the pleura and along the perilobular connective tissue At other times, however, the pleura may be covered with a layer of fibrin 1 to 2 cm thick and there may be anywhere from 1 to 20 litres of reddish-yellow or yellowish clear exudate in the pleural cavity (63, 97) After subcutaneous injection of infected lymph or virulent culture the lesion is one of extensive edema, the connective tissue is gelatinous and masses of clear, amber fluid are found along its extensions Histologically, K F Meyer (58) reported that thick rings of leucocytes and fibrin deposits around the interstitial blood vessels were rather characteristic According to Ziegler (104) the necrosis, interstitial perivascular and marginal organization processes, and parabronchial changes are diagnostically significant

In agalactia, the process in the mammary gland is that of an interstitial mastitis characterized by a disappearance of glandular tissue with invasion of young fibrous tissue Suppuration is extremely rare The eye lesion is that of an interstitial parenchymatous keratitis with infiltration of small cells and vascular proliferation in the thickened cornea In the joints the process is usually limited to congestion, cellular infiltration and thickening of the periarticular connective tissue without involvement of the synovia or cartilage, occasionally, however, there is proliferation, thickening, focal necrosis and cellular infiltration of the synovia and erosion of the articulating cartilage and more rarely still

there is formation of fungous polyps consisting of young connective tissue. As a rule, these lesions completely regress and ankylosis is very rare. An endarteritic process can be seen in the joint tissues (9).

The pathological changes produced by the Type A microorganism of mice vary with the site into which it is inoculated. Its multiplication in the peritoneum and pleura gives rise to no local lesions, but the toxin that is elaborated produces not only a distintegration of the posterior pole of the cerebellum but also intense degenerative changes without inflammatory reaction in the neopallial cortex, basal ganglia, and nervous part of the retina (74). When the joints are affected after intravenous injection the process is limited to the periarticular connective tissue and synovia (proliferation and subacute inflammation) without involving the articular cartilage. There is always complete recovery and no ankylosis. With the Type B microorganism, on the other hand, the pathological changes are limited to the joints and involve the capsule, synovia, articulating cartilage and subchondral bone marrow. The essentially proliferative character of the process is apparent as early as the second day after swelling of the joint is discernible and becomes more marked subsequently, affecting the capsule, synovia and perichondrium to such an extent that obliteration of the free joint-space may result. Actual destruction of cartilage has not been seen until about 4 weeks after intravenous inoculation, and at 7 weeks one may find the normal articulating cartilage replaced by undifferentiated cells which may be immature chondroblasts or osteoblasts. In a completely ankylosed joint, at about 5 months, one finds that considerable ossification has occurred in the distorted articulating surfaces which are joined by dense fibrous tissue (76, 78).

The pathological effects produced by the L_4 microörganism of the rat, either as the filtrable pyogenic agent or as the etiological agent of the spontaneous polyarthritis, appear to be quite different from those described above, and are influenced by the fact that the microoorganism has no special tissue affinities. Frank suppuration with the formation of an encapsulated abscess occurs at the site of subcutaneous injection in the rat, while in the mouse

massive edema develops involving the whole side of the animal, microscopic examination of which reveals extensive hemorrhagic necrosis with infiltration of polymorphonuclear leucocytes After intravenous injection "the suppurative lesions in the feet involved the soft parts only, the bone itself being spared though the marrow was thickly strewn with polymorphonuclear leucocytes" (101) In the spontaneous polyarthritis of rats the process appears to be similar and sometimes "while the joint is still swollen, the skin may ulcerate, and the limb below the joint may be gnawed off by the rat" (37)

EFFECTS OF IMMUNE SERUMS, VACCINES, AND CHEMOTHERAPEUTIC AGENTS

Wherever the effect of immune serums has been studied against *natural or experimental infections produced by members of the* pleuropneumonia group, the result has generally been that a distinct protective effect could be obtained when the serum was administered before infection but that even early after infection there was either no therapeutic effect or only a doubtful one Nocard, *et al* (64) showed this to be the case in pleuropneumonia bovis with hyperimmune bovine serum, and Dujardin-Beaumetz (30) reported a similar result with hyperimmune horse serum Carré (9) demonstrated the protective effect of hyperimmune serums prepared with agalactia "virus" and Bridré and Donatien (6) obtained similar results with cultures of the microorganism although they found it more difficult to protect against intra-articular than against subcutaneous inoculation, curative tests on a few animals during the first few days of the disease were without effect or inconclusive Sabin and Morgan (79) showed that potent immune rabbit serum which could completely protect mice against the polyarthritis produced by Type B and the nervous and arthritic manifestations of Type A, had little or no effect when administered after the first clinical signs of disease had appeared

Bovine pleuropneumonia is among the first diseases whose control was early attempted by protective vaccination The earliest successful results were obtained by subcutaneous injection

of infectious lymph (serous exudate) into the tail (97, 99) After cultivation of the microörganism the same results were obtained with living cultures, but in both instances the reactions were not only severe but sometimes fatal Recently, Purchase (73) studying control of the disease in Rhodesia found that while the 11th to the 45th generations of *in vitro* cultures were harmless to cattle, they gave rise to distinct immunity lasting for 2 years, and yielded fairly satisfactory results in the field, he also noted that vaccination was of no value when signs of disease had appeared Kurotchkin (51), however, reported that in the heavily infected regions of China a field test with 520 animals revealed that "whereas no arrest of epizootics occurred when attenuated culture was used, rapid eradication of the disease was obtained after immunisation with virulent culture" In agalactia, Bridré and Donatien (6) found that (a) living cultures could not be used because they produced the disease regardless of the route of inoculation, and noninfective doses did not give rise to immunity, (b) even after 100 passages *in vitro* the cultures could not be used because they had not completely lost their virulence, (c) various combinations of serum and cultures yielded no immunity if disease was prevented, and (d) no immunity was produced by cultures killed at 56° for one hour or by formalin It is of interest, therefore, that Sabin and Morgan (79) found that vaccination with concentrated suspensions of the mouse Type B microörganism killed at 50° (30 minutes) could protect the majority of mice against the experimental polyarthritis although it was practically ineffective when it was begun after the first clinical signs of disease had appeared

Effect of Chemotherapeutic Agents

In 1928, Bridré, *et al* (7) reported that the subcutaneous injection of the sodium salt of stovarsol (acetylamino-hydroxy-phenylarsonic acid) exerted both a preventive and curative action on agalactia contagiosa, as it occurs in nature and as it is produced experimentally in sheep and goats It is interesting that the reason these investigators chose stovarsol for this chemotherapeutic test is that the microörganism of agalactia is

stained by the same dyes as the protozoa but not by the usual (aniline) bacterial stains In 1938, Pigoury (69) indicated that even a single dose (0 75 gm) of stovarsol had a distinct therapeutic effect on the course of the mastitis and arthritis in 27 animals, while among 25 others treated with urotropine and in 5 untreated controls the disease pursued its normal course He also found that a single dose of stovarsol did not protect a well animal for more than a week Witt is quoted (38) as having observed a beneficial effect of arsenicals in bovine pleuropneumonia

Two organic gold compounds, aurothioglucose (solganal B) and aurodetoxin, were found capable of preventing the development of an arthritis in rats which was subsequently shown to be caused by a microorganism of the pleuropneumonia group (14, 19) When Findlay, *et al* (37) described the pleuropneumonia-like microörganism which causes the spontaneous polyarthritis of rats in London, they also confirmed the preventive effect of aurothioglucose on the development of the experimental disease In further chemotherapeutic experiments Findlay, *et al* (38) studied the capacity of various chemical compounds to *prevent* (a) the "encephalitis" which results from the intracerebral injection of the L_4, L_5, and L_6 microorganisms in mice, and (b) the "arthritis" which develops chiefly in the inoculated extremity following the injection of L_4, L_5, L_6, L_7 (which serologically is identical with L_4), and a guinea-pig strain of *Streptobacillus moniliformis* into the foot-pads of mice and rats The various compounds were administered subcutaneously immediately after the infectious agents and daily thereafter for 3 days The authors concluded that (a) *as regards prevention of the encephalitic syndrome and death,* sulfanilamide, sulfapyridine, and sodium salicylate were without effect, neoarsphenamine, acetarsol (stovarsol), and to a less extent tryparsamide were slightly active against L_5 and L_6 but not L_4, while aurothioglucose protected the majority of mice against all three, (b) *as regards prevention of the arthritis,* sodium salicylate, lithium antimonyl tartrate were without effect and neoarsphenamine had a slight effect when tested against L_7, while the organic gold compounds (aurothioglucose, sodium aurothiomalate, lopion, parmanil, and neosolganal) were highly

active against all the microörganisms tested, (c) "organic gold preparations are bactericidal to pleuropneumonia-like organisms both *in vitro* and *in vivo* "

The "arthritis" produced by these microörganisms developed in 2 days and treatment started at that time or later did not appear to result in a disappearance of the lesion in the subsequent 10 days The authors believed, however, that there "appeared to be some slowing up of the infective process, but since the swelling and arthritis are self-limiting and tend to disappear eventually in the absence of any treatment, it is not easy to judge how far solganal B affects the process once it is fully developed "

The curative effect of certain gold compounds was established in studies carried out by Sabin and Warren (82) on the experimental, proliferative chronic arthritis of mice produced by Type B They found not only that a curative effect could still be obtained when treatment was delayed as long as 3 weeks, but also that a therapeutically active gold compound had no apparent effect *in vitro* on the microörganism causing the arthritis Growth, even of minimal inocula, was not inhibited or prevented by concentrations of sodium aurothiomalate varying from 1 to 200 mgm per cent in the fluid media (a single dose of 2 mgm can cure mice), and microörganisms grown for several generations in the presence of gold did not lose the capacity to produce arthritis While the discrepancy between these results on the effect of gold compounds *in vitro* and those of Findlay, Mackenzie, and MacCallum may be due to the fact that different microörganisms were used, it is my opinion, rather, that the conclusion of the latter workers was based on insufficient data (which, as they indicated, they were prevented from enlarging by circumstances beyond their control) Neoarsphenamine, silver arsphenamine, bismuth subsalicylate, sodium salicylate and colloidal gold were without effect on the chronic arthritis in mice Sabin and Warren have used this experimental disease in mice in a search for chemotherapeutic agents with high curative properties and low toxicity, and have recently reported the preparation of a new compound, calcium aurothiomalate, which is practically nontoxic in mice and yet produces a curative effect after a single dose of 1 mgm (83)

It is of interest to note that in at least two diseases (agalactia contagiosa and experimental chronic arthritis in mice) caused by members of the pleuropneumonia group, chemotherapeutic agents possess a curative effect which is not exerted by the specific immune serums

POSITION OF PLEUROPNEUMONIA GROUP IN RELATION TO VIRUSES, RICKETTSIAE, COCCOBACILLIFORM BODIES, AND OTHER FILTRABLE AND NONFILTRABLE BACTERIA

For comparative purposes one may perhaps define the filtrable microorganisms of the pleuropneumonia group as polymorphic forms, which can (a) multiply in cell-free media and produce minute colonies (usually not exceeding 0 6 mm) on suitable solid media, (b) reproduce in several different ways, and (c) produce, as part of their life cycle, elementary bodies (usually 125–250 mμ in size) capable of reproducing the larger, more polymorphic and complex forms One may add that while they are best visualized tinctorially by the staining methods used for protozoa, and while in pathological material or in cultures containing a great deal of serum they are not revealed by gram-staining or the usual aniline dyes, they can, nevertheless, be so stained (especially in smears of centrifuged cultures) and are then gram-negative What they have in common, then, with the viruses is chiefly the small size of one phase of their life cycle The viruses, however, are characterized not only by their small size but also by the fact that they do not possess in their make-up the constituents which would permit them to exist as autonomous units of life, that is, they must depend upon some more highly organized living cell to supply the materials or functions by which they can multiply (or increase in amount) and thus perpetuate themselves It has occasionally been assumed that the very small size of even the larger viruses precluded their possession of enough of those enzyme systems which make possible the existence of independent living units Relatively, therefore, it is of interest that in the microörganisms of the pleuropneumonia group we have examples of minute particles, which, while not exceeding in size many of the typical larger viruses, nevertheless, possess the properties of

independent living units. And yet there appears to be, in addition to size, still another link to the viruses. For with certain members of the pleuropneumonia group, this property of independent existence may be only a matter of adaptation. I am referring to the Type A and Type B microörganisms of mice which in the animal body do not seem to be able to multiply until they locate in the right type of cell where they may be seen to proliferate in the cytoplasm. Upon being transferred to cell-free media, the assumption of independent life by these minute particles seems to depend upon the appearance of a special mechanism which may be so specific that after adapting themselves to the serum protein of one animal, they cannot survive when the protein of another animal species is substituted. (Personal observations on some strains of the mouse pleuropneumonia group.) Thus one finds in this group the minutest microbial units endowed with the living functions of independent growth and reproduction, a property which, in the present state of our knowledge, clearly differentiates them from the viruses.

Zinsser (105) defined the rickettsiae, as "minute bacillary or diplococcus-like organisms which are pleomorphic to the extent that range of size and shape may vary from that of minute, just-visible coccobacilli to longer filaments which sometimes approach bacillary size. Although not unstainable by ordinary methods of bacterial staining, as formerly supposed, they are best brought out by special methods—most useful among which are Giemsa and the stain of Castaneda." He further said that "the *Rickettsiae*, in spite of many claims to the contrary, have never been cultivated without the presence of living cells." More recently Zinsser and Schoenbach (106) indicated that the obligate intracellular parasitism of the pathogenic rickettsiae differed from that of the viruses in that multiplication of the former was favored by metabolically inactive cells. This obligate intracellular parasitism as well as the absence of the marked polymorphism thus readily differentiate the rickettsiae from the pleuropneumonia group.

In 1935, Nelson (59) reported certain minute *coccobacilliform bodies* which were associated with infectious fowl coryza, and in

later communications he described morphologically and culturally similar microorganisms as the cause of infectious catarrh of mice (60) and albino rats (62) The *coccobacilliform bodies* as they appear in exudates and in cultures, consisting of minced chick embryo suspended in Tyrode's solution, are gram-negative While originally, they do not grow on ordinary media with or without blood or serum, Nelson found that they are not obligate intracellular parasites, because they can grow in the Tyrode's solution extract of embryonic tissue, free of cells (61) Furthermore this diffusible growth factor withstands heating at 100° for 60 minutes Nelson also found that after many passages one strain of fowl coryza bodies could grow in the fluid blood at the base of an agar slant No colonies were seen on the agar but under similar conditions microörganisms of the pleuropneumonia group are also most unlikely to form colonies In describing the rat catarrh *coccobacilliform bodies*, Nelson (62) pointed out that they exhibited greater pleomorphism than those of fowl coryza, in cultures "at 48 hours it was customary to find small uniformly stained spherical cells, slightly larger ring forms with a central unstained area, short uniformly stained rods, short forms with a bipolar appearance, and small curved or spindle-shaped rods" The mouse catarrh bodies present in exudate could be filtered through a gradocol membrane with an A P D of 640 mμ (60) Nelson (62) further states that "it is recognized that there is a morphological similarity between the *coccobacilliform bodies*, particularly those of mouse and rat catarrh, and the organisms of the pleuropneumonia group" While the apparent ease with which the bodies are revealed by the Gram's method and the absence of colonies on solid media may be regarded as distinguishing characteristics, it would seem that not enough work has as yet been done by the methods employed in studying the microorganisms of the pleuropneumonia group to permit either inclusion or exclusion of the *coccobacilliform bodies*

As regards certain well-known filtrable bacteria such as the anaerobic *B pneumosintes* (*Dialister pneumosintes*), the members of the genus *Veillonella* including *Staphylococcus parvulus* (*Veillonella parvula*), *Staphylococcus* or *Micrococcus minimus*, etc , and

others, almost the only property they possess in common with the microörganisms of the pleuropneumonia group is their relatively small size, lacking altogether the capacity to give rise to the considerably larger and more complex polymorphic forms Among the other numerous species of filtrable, gram-negative, anaerobic bacteria found in the human nasopharynx, Olitsky (67) has described some as being pleomorphic, tenuous, vibrio-like and forming minute colonies on solid media Not enough is known about these microorganisms to permit their arbitrary exclusion from the pleuropneumonia group, and it may indeed be worth while to restudy them by the more recently developed methods Laidlaw and Elford (52) writing of the pleuropneumonia-like sewage microorganisms, stated "We do not consider that this group is related in any way to the 'filterable forms' of the pathogenic bacteria which have been described by many, e g , Hadley, Delves, and Klimek (1931), or Kendall (1931 and 1932) The existence of these filterable or 'virus' forms is still contested, but in any case they arise under ill-defined conditions or as the result of special treatment (Hadley *et al*), or on a particular kind of medium (Kendall) The 'virus' forms are said to multiply for a time, as such, but may, under appropriate conditions, revert to the larger form, which in turn, multiplies as such "

When one inquires in what way, if any, the microörganisms of the pleuropneumonia group differ from all the other bacteria, i e , the class of *Schizomycetes* one is confronted chiefly with questions concerning the extreme polymorphism and mode of reproduction On these questions there is not much uniformity of opinion at the present time As the name itself suggests multiplication among the *Schizomycetes* is characteristically by cell fission, with endospores "formed by some species of the *Eubacteriales*, [and] conidia by some of the filamentous forms " (2) Turner (96) discussing the microörganism of pleuropneumonia bovis in 1935 stated "What distinguishes it most clearly from other members of the class *Schizomycetes* is 1) its extreme pleomorphism, 2) the characteristic polygenethodism, or provision of alternative modes of reproduction, and 3) its peculiar

protean or amoeboid tendency to change its shape relatively quickly, which is seen nowhere else among the *Schizomycetes*" When Turner wrote this there were only two species in the pleuropneumonia group, and despite his appreciation of the fundamental difference between them and the *Schizomycetes*, he suggested that they be classified as a separate order under the *Schizomycetes* It seems to me, however, that if one accepts Turner's viewpoint as applying to the entire group, as I am inclined to do, one cannot classify these microorganisms under the *Schizomycetes* without modifying the definition of that class

CLASSIFICATION AND NOMENCLATURE

It is not without misgiving that I attempt to classify and name the ever-increasing number of microörganisms in this group That the job has to be done cannot be denied but it is doubtful that it can be done without having almost everyone else disagree with the result The very title of this review is in a sense misleading for it pretends to describe a pleuropneumonia group of microörganisms when, with one exception, they have nothing to do with pleuropneumonia One thing is certain, and that is that we cannot go on calling all the known, as well as yet to be discovered, microorganisms pleuropneumonia-like without twisting our tongues and confusing our minds

In the preceding section on the position of these microörganisms in the microbial world, I set forth the reasons for my belief that they belong in a class distinct from the *Schizomycetes* It is suggested, therefore, that a new class be established and that it be called *Paramycetes* Because I could not think of anything as short that would at the same time be descriptive of the cardinal characteristics of the group, and because para ($\pi\alpha\rho\alpha$) meaning beside or alongside suggests the position of this class as being close to the others, this name was selected to include all the microorganisms which because of morphology or mode of reproduction cannot be included with either the *Schizomycetes* or *Eumycetes* One order to be called *Paramycetales* is proposed and it is to be characterized by the properties which were previously

given as applying to all the microörganisms of the pleuropneumonia group Since the parasitic species are quite distinct from the saprophytic ones, it is suggested that two families be established family I *Parasitaceae* to include all the pathogenic and nonpathogenic microorganisms found in animals, all requiring a suitable amount of protein for growth, family II *Saprophytaceae* to include the nonpathogenic microorganisms, requiring no protein for growth, found in sewage, soil, etc Since the parasitic members are most clearly distinguished from one another, biologically and immunologically, according to the species of animal they inhabit and since a number of distinct species of these microörganisms have been found in the same animal, it is suggested that the names of the animals be incorporated in the names of the genera Thus

Family I *Parasitaceae*

- Genus I *Bovimyces* (microorganisms of cattle)
  - Species *Bovimyces pleuropneumoniae*, the etiological agent of pleuropneumonia contagiosa bovis
- Genus II *Capromyces* (microörganisms of goats and sheep)
  - Species *Capromyces agalactiae*, the etiological agent of agalactia contagiosa of goats and sheep
- Genus III *Canomyces* (microörganisms of the dog)
  - Species *Canomyces pulmonis* I, *Canomyces pulmonis* II — Distinct microörganisms found in the respiratory tract
- Genus IV *Murimyces* (microörganisms of rats, occasionally of mice)
  - Species *Murimyces streptobacilli-moniliformis*, usually in association with *Streptobacillus moniliformis* but occasionally independently (L_1)
  - *Murimyces pulmonis*, usually in association with certain lung lesions in the rat (L_3)
  - *Murimyces arthritidis*, etiological agent of spontaneous arthritis in the rat (L_4)
- Genus V *Musculomyces* (microörganisms found only in mice)
  - Species *Musculomyces neurolyticus*, produces an exotoxin with a neurolytic action especially on cerebellum (Type A)

Species *Musculomyces arthrotropicus*, has an almost exclusive affinity for joints giving rise to a chronic arthritis (Type B)
Musculomyces histotropicus, pathogenic, with broader tissue affinities (Types C, D, E)

When the existence of distinct species of similar microorganisms is established in man, guinea-pig, etc , the genera may be named *Hominomyces*, *Cavimyces*, etc The objection may justifiably be raised that it is uncommon to have genera of microorganisms limited to one species of mammal, but thus far that has been remarkably true of this microbial group In the saprophytic group only two distinct species are recognizable thus far on the basis of both cultural and immunological differences It is suggested that the genus be called *Sapromyces*, and that the species be names in honor of Sir Patrick Laidlaw who together with Elford first described these microörganisms

The proposed classification and nomenclature of the known microorganisms of the pleuropneumonia group may thus be summarized as follows

- Class *Paramycetes*
  - Order I *Paramycetales*
    - Family I *Parasitaceae*
      - Genus I *Bovimyces*
        - Species 1 *Bovimyces pleuropneumoniae*
      - Genus II *Capromyces*
        - Species 1 *Capromyces agalactiae*
      - Genus III *Canomyces*
        - Species 1 *Canomyces pulmonis* I
        - Species 2 *Canomyces pulmonis* II
      - Genus IV *Murimyces*
        - Species 1 *Murimyces streptobacilli-moniliformis*
        - Species 2 *Murimyces pulmonis*
        - Species 3 *Murimyces arthritidis*
      - Genus V *Musculomyces*
        - Species 1 *Musculomyces neurolyticus*
        - Species 2 *Musculomyces arthrotropicus*
        - Species 3 *Musculomyces histotropicus* (mixed species of many types)

Family II *Saprophytaceae*
Genus I *Sapromyces*
Species 1 *Sapromyces laidlawi* AB
Species 2 *Sapromyces laidlawi* C

In suggesting this classification I am not unmindful of Ledingham's (53) recommendation in 1933 that the microorganisms of pleuropneumonia and agalactia be grouped together merely as a new genus in the existing family of *Actinomycetaceae*, and of Turner's (96) proposal in 1935 of a new order which he named *Borrelomycetales* in honor of Borrel, who according to Turner first demonstrated the characteristic morphology Since Bordet (3) was the first to describe the complex morphology of the pleuropneumonia microorganism as Borrel et al (4) point out in their first communication, and since there already exists a genus called *Borrelia* in the family *Spirochaetaceae*, the name *Borrelomycetales* is an inadvisable choice If in the course of time the weight of evidence is in favor of these microörganisms belonging to the class of *Schizomycetes*, the remainder of this proposed classification and nomenclature might perhaps still be useful in distinguishing the various members of the so-called pleuropneumonia group

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PLATE 1*

Figures 1, 2, 9, 10, 11, 12, 13 are reproduced from Ledingham (53) and figure 3 from Klieneberger (48) The others are my own and with the exception of figures 4 and 16, have not been published previously

Fig 1 "Fully developed colonies of pleuropneumonia (strain 'Shanghai') and others which have not yet acquired the clear peripheral rings × 70" (J Path Bact)

Fig 2 "Colonies of pleuropneumonia Strain 'PP' × 70" (J Path Bact)

Fig 3 L_4 colonies after 10 days' incubation × 80 (J Hyg)

Fig 4 "X" colonies of Sabin and Johnson (81) grown from human tonsils × 150 (From Proc Soc Exptl Biol Med)

Fig 5 Type A microörganism of mice Colonies (3 days' incubation) photographed with transmitted light × 100

Fig 6 Same, photographed with oblique lighting × 100

Fig 7 Type B microörganism of mice Colonies (3 days' incubation) photographed with transmitted light × 100

Fig 8 Same, photographed with oblique lighting × 100

Fig 9 4-day culture of pleuropneumonia in serum broth, showing condensed rings and spherical elements Giemsa × 1500 (J Path Bact)

Fig 10 5-day culture of pleuropneumonia in serum broth, showing predominance of "elementary bodies" Giemsa × 1200 (J Path Bact)

Fig 11 Impression of 2-day old pleuropneumonia colony on serum agar showing "moniliform elements" and "yeast-like" bodies Giemsa × 2000 (J Path Bact)

Fig 12 Same "Active peripheral growth with pseudopodial budding of the large yeast-like bodies Giemsa × 2000" (J Path Bact)

Fig 13 Impression of 6-day-old pleuropneumonia colony on serum agar "Composite drawing of 'nucleated' bodies with chromatic elements surrounded by blue-stained sheath Giemsa × 1500 " (I have seen similar structures in cultures of the Type B microörganism of mice in the early passages after isolation from the animal body, they were present in fluid as well as solid media) (J Path Bact)

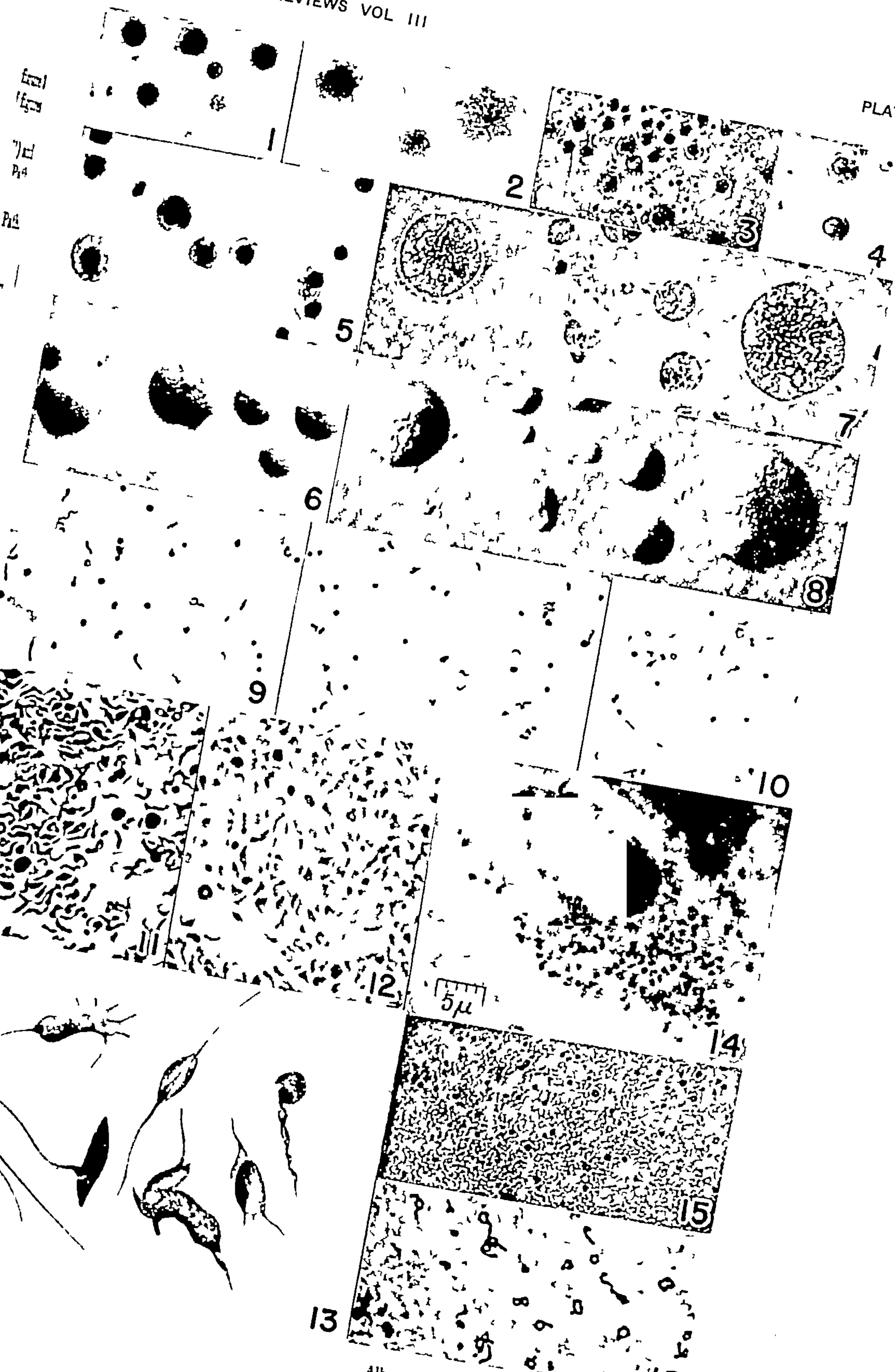
Fig 14 Mesothelial cell of peritoneum of a mouse inoculated with brain tissue infected with a Type A microörganism, that had been maintained by animal passage and had not been cultured *in vitro* Compare the typical forms of the microörganism seen in the cytoplasm of the cell with those shown in Fig 15 Giemsa × 1600

Fig 15 2-day-old culture in serum-glucose broth of the Type A microörganism of mice Note relatively simple morphology consisting of elementary bodies and rings exhibiting one or more dense bodies Giemsa × 1000

Fig 16 2-day-old culture in serum-glucose broth of the Type B microörganism of mice Note the more complex morphology especially the filaments "growing out" of the rings Giemsa × 1000 (J Bact , Ref 82)

* Please note how remarkably different are the forms seen in preparations from fluid cultures as compared with those found in impressions of colonies on solid media

PLATE 1



Albert

THE CHEMISTRY OF THE PROTEINS OF THE ACID-FAST BACILLI

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The protein components of bacteria are in most cases of great biological significance, and an understanding of their chemical compositions and properties is one of the pressing problems in bacteriological chemistry There is reason to expect that such knowledge will be of value in throwing light on the organisms themselves and perhaps also on some of the biological reactions that they elicit One aspect of the long study on the tubercle bacillus has been motivated by these considerations

Naturally, most such investigations have dealt with the proteins of the human type of the tubercle bacillus, while studies on the proteins of the other types and of other members of the acid-fast group have been fewer and generally less intensive As a result the advance in our knowledge has not been uniform, as will become evident from the following survey No attempt will be made to review the extensive literature dealing with the clinical tests or epidemiological surveys that have been carried out with the "Purified Protein Derivative Tuberculin" or other preparations We shall consider here only those studies that have dealt specifically with the differentiation of the proteins from different acid-fast bacilli, or those that have led to the chemical characterization of these proteins

In the beginning of our accurate knowledge of tuberculosis, with the discovery of the causative bacillus in the disease, Koch's experiments in 1891 (35) led him to believe that the substance responsible for eliciting the specific toxic effect in the diseased animal was of protein nature, and Hammerschlag (23) was among

[1] Aided by grants from the Committee on Medical Research of the National Tuberculosis Association

the first to identify protein as one of the constituents of the tubercle bacillus Weyl (93) in the same year, also isolated a protein and considered it to be a mucin, differing, however from other mucins in that on heating with dilute acid, a reducing substance was not split off

About the same time Kuhne (37), well known for his fractionation studies on proteins and their decomposition products, made exhaustive investigations on the constituents in Koch's tuberculin, but the "akroalbumose", a propeptone which he found and which was precipitable by acetic acid, could be identified also in the peptone used in the medium In later studies, however, he made a tuberculin by growing tubercle bacilli upon a synthetic medium and was able to show the presence of a protein, which must have been a metabolic product of the bacillus He used the alcohol precipitation method of Koch and isolated an albumose mixture which contained the whole of the active material Hofmann (28) obtained six protein fractions from extracts of tubercle bacilli, two of which he called albumin and globulin

Nucleoprotein Theory Levene, however, in 1898 made the following statement (39) "The body substance of tubercle bacilli does not contain any proteids of albumin nature, that as the nucleus of cells it consists mostly of nucleoproteids, that one of the nucleoproteids differs from all the other nucleoproteids inasmuch as it is not precipitated by magnesium sulfate (and in this point resembles nucleo-histon), and does not give the [biuret] test, that it contains nuclein or nucleins as such" He obtained three proteins with coagulation points at 50–64°, 72–75°, and 94–95° In later experiments he used a medium free of protein, in which the source of nitrogen was asparagine and the source of carbon was mannitol and glycerol

In the same year Ruppel's (57) extensive researches appeared At first he fractionated tuberculin by alcohol precipitation, then by precipitation with saturated sodium chloride, and finally the filtrate with acetic acid The chief amount of material, however, remained in solution after this precipitation and could be obtained by dialyzing the solution, evaporating and again precipitating with alcohol The final product did not precipitate from aqueous

solution with saturated sodium chloride, but was completely precipitated by ammonium sulfate and he therefore considered it to be mainly a deuteroalbumose

By dialyzing the original tuberculin before fractional precipitation he could remove a heteroalbumose fraction Furthermore, he studied innumerable methods of extracting the bacilli and finally used the method given by Koch of completely disintegrating the bacilli by mechanical grinding until no intact bacilli remained By this method half of the bacilli by weight went into opalescent solution The solution gave with acetic acid a considerable precipitate which was insoluble in excess of the acid, but soluble in dilute alkali This precipitate contained four per cent phosphorus, gave a positive biuret test, but negative Millon and xanthoproteic reactions From this product he obtained by Kossel's purification methods a product which fulfilled the specifications of a protamine For example, it existed as a sulfate of a basic compound, it was phosphorus-free and precipitable by sodium picrate in neutral solution, it gave only the biuret reaction, and finally it precipitated proteins in ammoniacal solution Ruppel named the product "Tuberkulosamin" and claimed it was bound in the tubercle bacillus to a nucleic acid, which he was also able to isolate, some of which existed in the free state This acid contained 9 4 per cent phosphorus, and was named "Tuberculinsaure "

Klebs (34) and others were also of the opinion that there existed in the tubercle bacillus a nuclein and that it was responsible for the specific tuberculin activity This hypothesis was further supported by Baldwin and Levene (40) on the basis of their results showing that trypsin more easily destroyed the tuberculin potency than did pepsin

Non-Nuclein Nature of Active Principle Based on Reaction to Enzymes Löwenstein and Pick (44), however, claimed that the tuberculin product was destroyed by digestion with pepsin as well as with trypsin and had, therefore, the characteristics of a polypeptide Danièlopolu (14, 15), Pfeiffer, Trunk and Leyacker (54), Mueller (53) and others also found that pepsin as well as trypsin destroys the active substance Seibert (62) studied the

effect of pepsin, trypsin and erepsin upon a purified, water-soluble, non-coagulable tuberculin protein and followed, simultaneously, the breakdown of the protein molecule by chemical analyses and the loss in activity It was concluded that when the whole protein molecule is attacked, as by pepsin in acid or trypsin in alkaline solution, activity is lost, whereas when the peptide bonds in the proteose molecule are attacked, as by erepsin, no loss in activity occurs In view of the recent isolations of highly purified, crystalline enzymes, it would be of value to restudy this problem

This contention of Ruppel concerning the nucleoprotein nature of the active tuberculin fraction was further refuted by Toenniessen (88) His preparation, which he called "Tebeprotin," was made by heating the bacilli in dilute mineral acid, extracting with alkali, filtering through a Berkefeld candle and then precipitating and reprecipitating with acetic acid It contained 12 3 per cent nitrogen, no phosphorus, no purine bases, and gave a negative Molisch reaction but positive biuret and Millon reactions, 0 1 mg of "Tebeprotin" had a tuberculin potency equivalent to 10 mgm. of Old Tuberculin

Other Theories as to the Nature of the Active Principle In addition to the opinions cited above, the debate as to the nature of the active substance in tuberculin was carried on by the following investigators Much (52) believed it was associated with lipoid Bieling (5) claimed it was not a single pure substance, but something which readily adsorbs to numerous other substances, including protein, protein derivatives, kaolin, aluminum hydroxide, silicic acid, etc Jastrowitz and Weinberg (29) and Sandor (59) were of the same opinion Later Boquet (8) thought it accompanied something of proteose nature, and so also did Kallós and Hoffmann (33) Some of these views were due largely to the experiments on dialysis of tuberculin

Dialysis of the Active Principle On the subject as to whether or not the active principle dialyzes the views have been numerous Zieler (95), Danièlopolu (13), Löwenstein and Pick (44), Zinsser (96), LeGuyon and Albert-Weil (38), and Maschmann and Kuster (46), among others, claimed that the active principle did

dialyze through membranes of viscose, collodion, vegetable parchment or fish-bladder On the other hand, Ruppel (57), Marie and Tiffeneau (45), Selter and Tancrè (82), Seibert and collaborators (61, 65, 66), Tytler (89), and Kallós and Hoffmann (33) maintained that the active principle could be held back on dialysis and that it could be fractionated and even concentrated in this manner, provided a suitable membrane was employed In actual fact, pressure-dialysis, or ultrafiltration is one of the important steps recently used on a commercial scale in securing the active principle in concentrated form before its final purification

Obviously membranes of different porosities were used by the different investigators and the matter resolves itself, not so much into the question as to whether the active principle of tuberculin does or does not dialyze, as into the question of how small a molecule can possess the tuberculin activity and also as to whether this activity can accompany molecules of different sizes That the activity does accompany molecules of different sizes is the prevailing opinion at the present time, and much evidence has accumulated to show that this is so This will be discussed later

Association of the Active Principle with Normal Protein Fractions In addition to the literature so far cited, mention should also be made of the following studies, all of which show that tuberculin activity accompanies fractions which could be considered to be more or less normal proteins Half-saturation with ammonium sulfate, as well as complete saturation, precipitated active protein fractions (63) from tuberculin filtrates obtained by growing tubercle bacilli on synthetic medium It was found, furthermore, by Seibert (64, 67) that a small amount of a protein with high tuberculin activity could be crystallized at an optimum pH of 4 9, by Hopkins' method for crystallizing egg albumin Very fine needles formed and they usually grouped themselves into burrs In one experiment, in which the protein was redissolved and recrystallized fourteen times, the potency was increased rather than decreased

Masucci and McAlpine (47) made a preparation, called MA-100, which was obtained after precipitation eight times by half-

saturation with ammonium sulfate, once with five volumes of 95 per cent alcohol at pH 4 7, four times at pH 4 7, and finally hydrolysis with barium hydroxide Later a very simple method for obtaining the active protein from tuberculin filtrates, in powder form, in which the potency and analyses were easily duplicated, was given by Seibert and Munday (70) This product was made by concentrating large quantities of filtrate from tubercle bacilli grown on synthetic medium by ultrafiltration and then precipitating once with trichloracetic acid and removing the trichloracetic acid with ether This product, called TPT, was used extensively in many researches

Gough (21) obtained an active preparation from tuberculin filtrate by first removing a precipitate at about pH 5 0 and then adsorbing the active substance on benzoic acid, from which it was freed with acetone Boquet and Sandor (7) precipitated an active fraction by means of phosphotungstic and sulfuric acids and then freed it by means of a saturated solution of baryta Gozsy and Vásárhelyi (22) tried to increase the degree of purification of a preparation already purified, by means of chromatographic adsorption, but were not able to do so and this fact they claimed argued for the purity of the original material However, Seibert, Pedersen and Tiselius (78) had shown that fractions obtained by chromatographic adsorption and elution at pH 8, gave asymmetrical curves when studied in the ultracentrifuge, indicating more or less molecular heterogeneity of the isolated fractions It is probable that the same phenomenon of interaction of components was in effect as was shown later by Seibert and Watson (81) to be so prominent in their attempts to purify the polysaccharides of tuberculin For example, it was not possible to remove the nitrogenous impurities (protein and nucleic acid) to less than about one per cent nitrogen from one of their polysaccharide fractions by exposure to an electrophoretic potential gradient of 3 7 volts per cm (about 400 v and 17 ma) for 114 hours On the other hand, another polysaccharide fraction was readily purified to a content of 0 2 per cent nitrogen in half the time under identical conditions

Active Protein from Bacillary Extracts Numerous prepara-

tions, considered to be protein, have been isolated from extracts of tubercle bacilli. Coghill (11) obtained an active water-soluble protein from bacilli previously defatted by repeated extraction with cold ether, and also a less active fraction by extracting the residue with alkali. The proteins were precipitated with acetic acid. Dienes, *et al.* (16) used aqueous and weak alkaline extracts of tubercle bacilli and precipitated the proteins with sodium sulfate or acid and heat. Later Dienes (17) fractionally precipitated the bacterial extracts at different hydrogen ion concentrations obtained by adding increments of hydrochloric acid. He thought he could detect by means of the precipitin reaction that the proteins obtained from the weak alkaline extracts were different from those obtained from the aqueous extracts. Tytler (89) extracted ground dried bacilli with acetone and then with water at 37° for 2 to 6 weeks. The protein was then precipitated from the aqueous extract with acetic acid at pH 3.8 to 4.0, redissolved at pH 5.5 to 6.0, reprecipitated with ammonium sulfate, dialyzed and dried under high vacuum from the frozen state.

An attempt to cause less denaturation of the bacilli during the defatting process was undertaken by Gough (21). He used Hardy and Gardiner's (25) method of extracting an ice-cold suspension of living tubercle bacilli, grown on synthetic medium, with a large excess of absolute alcohol at −10°, and then replacing at −3° the solvent with successive changes of mixtures of alcohol and ether containing increasing proportions of ether up to anhydrous ether. The idea that this procedure does leave proteins unharmed, however, has been questioned by McFarlane (50), since he found fundamental changes in the physical state of serum proteins dried in this manner.

Heidelberger and Menzel (26) also attempted to isolate the proteins of the bacillary bodies in undenatured form. They used living bacilli which had been frozen and dried in vacuum, and killed them by immersion in cold acetate buffer at pH 4.0 for 30 days. After this the cells were extracted four times in cold redistilled acetone, three times with purified anhydrous ether, dried *in vacuo*, ground for ten days, reextracted two more times with cold ether, and dried. The cell residues were then suc-

cessively extracted with buffers ranging from pH 4 0 to 11 0, and then finally in 0 1, 0 2 and 0 5 normal sodium hydroxide at room temperature Each fraction was then precipitated with acetic acid to maximum flocculation and repurified several times Among the many fractions obtained they identified two antigenic components, and finally, by subfractionating each fraction, they found at least three components

Search for Albumin and Globulin Attempts to identify typical albumin and globulin fractions in the tuberculin filtrate or in the bacillary extracts have also been made As early as 1894 Hofmann (28) classified two of his six fractions from bacillary extracts as albumin and globulin, the former being soluble in water and the latter in weak acid Seibert's (67) method of crystallizing a protein from the tuberculin filtrate would naturally classify it as an albumin, and furthermore, no significant protein fraction was ever found in the unheated culture filtrates which would precipitate out on dialysis against water Coghill (11) looked for the two types of protein in his bacillary extracts, and found a water-soluble fraction, precipitable with acetic acid, which he classed as an albumin But he could find no fraction which corresponded to globulin, since a 5 per cent sodium chloride extract yielded a protein fraction from which nothing would precipitate on dialysis against water

Gough (20) obtained protein fractions from his bacillary extracts which precipitated at half and complete saturation with ammonium sulfate, as globulins and albumins are supposed to do, and he called them such, even though the globulin did not precipitate from aqueous solution on prolonged dialysis against water The two fractions had distinct properties, indicating that a separation of different proteins had really been made, even though the tuberculin activity of both of them was about equal The albumin fraction contained more carbohydrate in definite association, more phosphorus, and retained its precipitating power after treatment with 0 5 per cent sodium hydroxide, in contrast to the globulin fraction

Protein Nature of the Active Principle The study of the proteins produced and released by the tubercle bacillus has been

chiefly, as noted above, the story of a search for the active principle of tuberculin In practically all of the studies cited, special attention has been given to the activity of the preparation and when a protein fraction appeared to be inactive or less active, interest in it was usually lost It is obvious that the activity accompanies fractions which can be isolated in many different ways, and the number of preparations mentioned in this paper is only a fraction of the list of tuberculin products offered for therapeutic and diagnostic use (4) Excellent reviews are given by Wells and Long (92), by Long (42), and by Calmette (9)

One is much impressed with the high degree of stability of the active principle even under the drastic treatment often used, and it is not easy to find in the realm of known biologically potent soluble proteins one with such a remarkably high degree of stability In this respect investigators in this field are fortunate However, more careful studies of this protein do not show that it is really so constant in regard to its physical properties, since fractions with innumerable different combinations of properties can be obtained from the same solution, simply by varying the method of isolation

All of these facts indicate that the biological potency must be inherent in some small, relatively stable part of the protein molecule, and many changes can be effected in the molecule as a whole, before there is recognizable loss in the biological activity With such an explanation all the different views can be brought into harmony

That the activity is associated with protein or some part of the protein molecule can no longer be doubted when one considers the evidence To summarize, the facts are as follows

1 Coagulable protein appears in the culture medium at about the same time as tuberculin activity
2 Protein precipitants carry down the activity along with the protein
3 When the protein fractions are purified the content of nitrogen parallels the potency
4 Pepsin and trypsin destroy the potency simultaneously with the breakdown of the protein molecule

5 The potency accompanies the crystallized protein and even has been shown to increase during 14 recrystallizations
6 When protein precipitable by trichloracetic acid is found in tuberculin ultrafiltrates, there is tuberculin activity also in these filtrates Otherwise, there is never more than a trace of potency
7 The potency accompanies protein molecules of different sizes which have been shown to be practically homogeneous in sedimentation and diffusion These will be discussed later
8 The active principle migrates with the protein in electrophoresis

Molecular Size of Active Principle That there may be potent protein molecules of different sizes, or that there may be potent molecules of different sizes which represent consecutive breakdown stages in the protein molecule was the logical suggestion from the apparently conflicting opinions in the literature Experiments with fractional ultrafiltration through graded membranes (69) also indicated that this was true An attempt to investigate this suggestion was made in 1933 (73) with the physico-chemical technics then generally available, and it seemed as though the potency could accompany molecules ranging in size from about 2000 to 34,000 However, a much more accurate and comprehensive study of this problem was made in Professor Svedberg's laboratory, Upsala, Sweden, by Seibert, Pedersen and Tiselius (78), using the precise ultracentrifuge, diffusion and electrophoresis technics there available These studies led to the conclusion that practically homogeneous fractions with molecular weights of 16,000 and 32,000 could be isolated from tuberculin, and both of them were highly potent A molecule even as small as 9000 had some potency Recently X-ray diffraction patterns have been obtained on some of the homogeneous proteins (85)

Previously it had been shown (71) that the molecule with 16,000 molecular weight did not stimulate the production of antibodies (precipitins), nor elicit a typical Arthus reaction when repeatedly injected into normal rabbits, whereas the molecule of 32,000

molecular weight was highly antigenic The smaller molecule had been isolated from heated tuberculin, like Old Tuberculin, and the larger one came from unheated culture filtrate Moreover, the small molecule became antigenic after it was adsorbed to aluminum hydroxide and charcoal (76) indicating that it was acting like a haptene This phenomenon seemed to indicate that we were dealing not with an irreversible breakdown of an antigenic whole protein molecule to a proteose, with loss in antigenicity, but possibly with a situation such as was assumed earlier (78), namely, that the potency might be inherent in a small unit molecule, similar to one of Svedberg's protein units of 17,000 molecular weight and that when two or more of these aggregated, the larger molecule would become antigenic There would be no difference in the nitrogen content of the two products

However, recent researches (80, 91) have shown that tuberculin protein molecules even smaller than 17,000 molecular weight have considerable antigenicity Since one of these, which has been studied by electrophoresis, has been shown to be somewhat heterogeneous and to consist of at least two components, the relationship of molecular size and antigenicity is still not entirely clear

Theory of a Reversibly Dissociable Component System It is obvious, however, that the system of tuberculin protein molecules under consideration is extremely complex The results so far available might suggest a reversibly dissociable component system such as postulated by Hardy (24), Sørensen (83), and Block (6) for some of the well-known proteins According to these investigators the proteins which one isolates from whole serum as definite chemical entities by simple chemical treatment with neutral salts, do not exist as such in the serum Whole serum protein is electrically inert, according to Hardy (24) and Tiselius (87) It may be considered as a system of reversible components so combined in its natural environment as to act as a single substance, but which can be readily dissociated into fractions whose composition and properties depend upon the degree of dilution and the reagents used Sørensen (83) stated that "Within each complex all the atoms or atom groups are inter-

linked by main valencies, whereas the various complexes of components are reversibly interlinked by means of residual valences " Thus if one of the components contained a high proportion of a certain amino acid, a fraction could be isolated which would contain a very high percentage of this particular amino acid, depending upon the method used for isolation and the amount of rearrangement of the co-precipitation systems For example, Block (6) obtained by fractionating the whole serum with neutral salts, different proteins which varied from 4 to 39 per cent in their content of lysine A good review of these hypotheses and the available evidence for them can be found in Schmidt's book (60)

It is true that innumerable tuberculin protein fractions can be isolated which vary in the proportions of protein, carbohydrate and nucleic acid components and also in their potency, toxicity and antigenicity Moreover, fractions with rather definite proportions of these components and constant biological properties can be repeatedly produced if the same method is used, at least from the same lot of raw tuberculin It is clear also that the biological properties mentioned are not dependent upon the presence of carbohydrate or nucleic acid, except insofar as a question of contamination or dilution may be concerned, since highly potent protein fractions demonstrated to be almost free of these constituents have been isolated (79) Thus we cannot consider the active principle to be a true nucleoprotein or a mucoprotein even though the potency may be present in such molecules It would seem that the potency of any fraction isolated depends upon the inclusion within its complex of a particular protein component that contains the specific group responsible for the tuberculin potency It is likely that this component is present in considerable proportion, since it is difficult to isolate completely inactive carbohydrate, nucleic acid or protein fractions from the tuberculin, and relatively easy to secure highly active tuberculin preparations with small differences in potency

The problem of isolating and identifying the specific component in the tuberculin protein-complex, therefore, becomes even much more complicated than in the case of serum, because of the

presence of the nucleic acid and polysaccharide components in addition to the proteins, of which it is still not certain how many exist

Amino Acid Analysis of the Proteins The question as to the existence of such a reversibly dissociable system in tuberculin protein fractions could possibly be answered by making comparative amino acid analyses on the purified protein preparations An attempt was made to do this by Seibert and Munday (74) in 1933, and there proved to be very much more total basic nitrogen in the small molecule (SOTT) than in the large molecule (TPA) However, not too much significance can be placed on these results since satisfactory micro-methods were not then available to show that the SOTT contained much more nucleic acid than the TPA, and this may have accounted for the high basic nitrogen Moreover, the presence of polysaccharide seriously affects the accuracy of the results The difficulty in an adequate study of this problem is the securing for analysis of sufficient quantities of the fractions really freed of nucleic acid and polysaccharide and molecularly homogeneous, such as those isolated and proved to have molecular weights of 16,000 and 32,000

Analyses of the amino acid content of the proteins isolated from extracts of tubercle bacilli have been made by Tamura (86), Johnson and Brown (31), Johnson and Coghill (32), Campbell (10) and Popper and Warkany (55) Tamura emphasized the high content of phenylalanine, and Johnson and Coghill emphasized the high content of hexone bases and low content of cystine Popper and Warkany found 1 1 per cent typtophane and 1 4 per cent tyrosine and pointed out that these constituents varied from the quantities usually found in animal and plant tissues

Denaturation of the Tuberculin Protein The exact composition of the active tuberculin protein complex becomes further complicated by the question of denaturation In addition to the supposition that the potency of a fraction may depend upon the proportionate amounts of active protein, inactive protein, polysaccharide and nucleic acid present, there is also the possibility that some denaturation of the active protein may have taken

place The degree of this denaturation may vary with different preparations according to their treatment

Table 1 is a compilation of data on a selected number of fractions studied at various times, and illustrates that the potency is not dependent upon the amount of nucleic acid or polysaccharide present, since large or small quantities of these impurities may be found in preparations with potency one-half or twice as great There is also no definite correlation with the antigenicity

TABLE 1

A comparison showing the lack of correlation between nucleic acid or polysaccharide content of certain preparations and their potency or antigenicity

PREPARATION	NUCLEIC ACID	POLYSACCHARIDE	RELATIVE TUBERCULIN POTENCY	RELATIVE ANTIGENICITY
	per cent	*per cent*		
TPA-30 K	0	2 0	*	Good
Rx	5 1	7 2	x	
98	27 7	32 6	x	
63	3 0	4 4	2 x	Moderate
19	21 8	24 0	2 x	Poor
65	0 8	4 8	4 x	Moderate
R5	21 2	20 1	4 x	Moderate

* The potency is not recorded, since it was greater than 2 x (x stands for the lowest activity recorded above) at 24 hours but less at 48 hours, indicating a more typical anaphylactic type of reaction Preparation TPA-30 K (considered to be highly antigenic) was isolated from an unheated tuberculin filtrate, while all the others were obtained from heated fractions

of the fractions It would seem that these biological properties are more dependent upon the type of protein components present, and possibly the extent of their denaturation

In the case of one "Purified Protein Derivative Tuberculin," a fractionation could be made (78) into three protein components of quite different properties For example, one (Fraction b3) was highly potent, a poor antigen, fairly homogeneous in ultracentrifugal sedimentation and in diffusion, with a molecular weight of about 16,000, and a frictional ratio of 1 9, indicating some dissymmetry of the molecule

There was present also a small amount of molecules about half this size, (Fraction b3b), which were less active and apparently homogeneous in the ultracentrifuge The third component (Fraction b2) occurred in considerable quantity, behaved like very heterogeneous, thread-like or hydrated molecules in sedimentation and diffusion, and was less active This fraction dissolved with difficulty in buffer at pH 8 0, swelling at first and then showing indication of gelling There is reason to believe that this reaction indicates the presence of denatured protein molecules Thus varying proportions of these three components would give products with varying potencies and physical properties

A similar denaturation occasionally occurs during concentration of tuberculin on ultrafilters, as evidenced by the formation of a gel on the membrane This gel has been shown (78) to consist of particles of many sizes and even of huge dimensions, with sedimentation constants as high as $S_{20} = 46$ It is usually less soluble and sometimes insoluble Furthermore, these gel fractions are always less potent

It is quite probable, therefore, that at least one form of denaturation of the tuberculin protein may occur first through a splitting or opening of the unit molecule with considerable loss in potency and exposure of extra polar groups, through which a polymerization of these elongated molecules may take place to form long thread-like structures Since the titration curves (78) indicate that the extra exposed groups may be ones which dissociate at about a pH 5 0, it is possible that the imino group of histidine may be important in determining the potency of tuberculin The investigation into the question as to which groups of the molecule are important in determining the tuberculin activity has of necessity waited upon the availability of preparations with molecules of relative homogeneity

"Purified Protein Derivative Tuberculin" As stated earlier, the study of the tubercle bacillus proteins has always been a search for the active principle and methods for isolating it in pure, stable and highly potent form for use in the diagnosis of tuberculosis From this standpoint, the search can be considered to have culminated at the present time in the production of "Purified

Protein Derivative Tuberculin," which has been used extensively in different parts of the world and which has been accepted as a standard tuberculin. Its preparation by means of trichloracetic acid, its properties, and some clinical results obtained with it were given in 1934 (75). It was a powder, whose potency has remained stable for at least five years and which could readily be made into quantitative solutions. It was relatively non-antigenic in comparison with the unheated protein.

Further study by means of spectral absorption (84) and electrophoresis (78) revealed the fact that this preparation still contained considerable nucleic acid and polysaccharide and that the amount varied with different preparations, according to how much was present in the original Old Tuberculin from which it was isolated. It was found also (79) that the nucleic acid migrated in electrophoresis along with the protein as a single component at pH numbers lower than 5.0, but readily separated and travelled with a much greater velocity at more alkaline levels. This suggested that a product with a lower content of nucleic acid might be obtained by precipitating the protein at pH 7.0. Therefore, saturated ammonium sulfate which had previously been neutralized with disodium phosphate was used as the precipitant.

Furthermore, other modifications were later introduced into the process, with the object of making the procedure less drastic and thus of decreasing the amount of denaturation of the protein. For example, the culture was heated in the Arnold sterilizer but the prolonged concentration on the steam-bath was eliminated, and the rest of the procedure was carried out at 5°. The final product was dried from the frozen state instead of by means of ether. It proved to be twice as potent as previous preparations. A very large quantity of the new "Purified Protein Derivative" was made by this method for use as a new official standard for tuberculins. Its preparation was reported by Seibert and Glenn (80). Satisfactory "Purified Protein Derivative" preparations for use in diagnosis made by these methods or slight modifications of them have been reported by Jensen, *et al.* (30) in Denmark, by Doig, *et al.* (18) in England, by Wong and Chu (94) in China, by Vásárhelyi and Gözsy (90) in Hungary, and by Leyva (41) in the Philippines.

CHEMICAL AND IMMUNOLOGICAL STUDIES ON PROTEINS OF OTHER ACID-FAST BACILLI

In comparison with the human type tubercle bacillus, no other group of acid-fast bacilli has received as much study In fact, very little experimental material is available Tamura (86) compared the analyses of the amino acids in *Mycobacterium tuberculosis* (human type) with those in *M lacticola perrugosum* and found lower contents of *l*-phenylalanine and valine but higher arginine and *l*-proline in the latter

One of the most interesting studies was that of Coghill and Bird (12) on the protein of *M phlei* (Moeller) They found marked differences in the properties of this protein and that isolated similarly from the (H37) human strain of tubercle bacillus For example, with the protein of *M phlei*, no precipitate occurred even in concentrated solutions with acetic acid, and only about one-third as much nitrogen appeared in the aqueous extract or in the 0 5 per cent sodium hydroxide extract as with the protein of the human tubercle bacillus However, 14 times as much of the water-soluble nitrogen in the *M phlei* fraction was protein nitrogen as in the case of the tubercle bacillus Thus, definite differences were demonstrated in the proteins of these two bacilli as well as in their nucleic acids The nucleic acid of the human type tubercle bacillus contained thymine, whereas that of *M phlei* contained instead uracil and methyl-cytosine

Renfrew (56) found that less water-soluble and less alkali-soluble protein as well as less polysaccharide existed in the avian tubercle bacillus than in the human strain

Since immunological methods are as a rule very sensitive, some serological studies have been made in the hope of differentiating the proteins of the acid-fast bacilli Serologically by means of the agglutination test, Koch (35) had shown that there is a relationship between the human tubercle bacillus and a large number of other acid-fast organisms, including some from cold-blooded animals, and that this relationship did not exist with other pathogenic bacteria Recent work has shown that there is probably a serological group-specificity due to the polysaccharides of the acid-fast bacilli, whereas a type-specificity is apparently due to the proteins in these bacilli For example, the proteins prepared

from cultures of a human, bovine and avian type tubercle bacillus and a timothy hay bacillus by precipitation with ammonium sulfate or trichloracetic acid (68) could be differentiated by means of the precipitin reaction The specific antigen always gave the highest titer, indicating a definite difference in the chemical composition or structure of these proteins The fact that there were some very definite cross-reactions indicates that there are probably certain chemical groups common to the proteins in the different acid-fast bacilli The human and bovine type proteins were the most difficult to distinguish, although even here there was some difference Later studies were made by Seibert (77) on the potency, precipitin reaction, and acid-base combining capacity of proteins made identically by the trichloracetic acid precipitation method (TPT) from culture filtrates of five different strains of human tubercle bacilli in comparison with the proteins from a bovine, an avian, a timothy hay and two leprosy strains The potencies of the five human strain proteins differed only slightly in some cases Serologically they were identical The acid-base combining capacity curves showed a much closer correspondence between the five proteins from human type tubercle bacilli than between them and the proteins made from the other types of acid-fast bacilli In view of more recent work, these last experiments should be repeated on fractions more pure with respect to nucleic acid contamination, since the acid-base combining capacity of the latter acid would markedly influence the shape of the curve Henderson (27) utilized some of the same methods for differentiating 27 strains of acid-fast bacilli, among which were 16 isolated from patients with leprosy, and he was able to make definite groupings, based upon the cross-reactions

The precipitin test was also used successfully by McCarter, Kanne and Hastings (49) for distinguishing between the proteins of the human, bovine, avian, and two saprophytic acid-fast bacteria isolated from the cow and hog Protein from Johne's bacillus was also distinguishable from all of these except the avian type Similar differentiation of the different acid-fast bacilli was made by Menzel and Heidelberger (51) They isolated protein fractions from the living cells of *M phlei*, and the

avian, and bovine tubercle bacillus by the same method which they had previously used on the human type Corresponding fractions were distinguishable from each other by means of the quantitative precipitin reaction, but the human and bovine proteins were very closely related

Potency tests also showed some difference in the proteins from different types of tubercle bacilli Seibert and Morley (72) precipitated by means of trichloracetic acid the proteins from the tuberculins of three different human strains, one of which was an extremely avirulent type, and they could not distinguish between them in potency when tested in tuberculous guinea pigs They did, however, find a difference between the potencies of the respective proteins made similarly from bovine, human, and avian tubercle bacillus filtrates, with decreasing potency for tuberculous guinea pigs in the order mentioned This has recently been confirmed Purified protein derivatives were made by the recently modified technic referred to above, from bovine, human and avian tubercle bacillus filtrates, the protein from the bovine type proved to be much more potent than that from the human type, and the latter was more potent than the protein from the avian type when tested intracutaneously in human beings. Moreover, the skin reaction with the avian protein was different. The area was soft and diffuse and usually did not reach the degree of induration or severity characteristic of the typical tuberculin reaction, except when the reactions were large

Some interesting results obtained with the avian purified protein derivative in several series of intracutaneous tests on students have been reported by McCarter, Getz and Stiehm (48) In their study a considerable number of students, especially those coming from farms, reacted to the avian but not to the human purified protein derivative No student with active tuberculosis failed to react to the human purified protein derivative Protein from the smegma bacillus filtrate gave results somewhat like those with the avian bacillus protein, and that from the bovine bacillus filtrate reacted like the purified protein from the human bacillus filtrate

A purified protein derivative prepared from a culture filtrate of

the timothy grass bacillus gave reactions (43) in subjects who required comparatively large doses (0 005 mgm) of the analogous protein from the human bacillus to cause a reaction Smaller doses, equivalent to the usual first dose (0 00002 mgm), gave no reactions

Similar intracutaneous cross-reactions had been found by Fenger, *et al* (19) to be caused by the proteins from various acid-fast bacilli Their fractions had been made from unheated culture filtrates and were purified by means of ammonium sulfate (MA-100). The amounts required to produce reactions varied with the source of the preparation and were as follows 0 0001 mgm of bovine, 0 0005 mgm of human, 0 001 mgm of avian, and 0 01 mgm of *M phlei* Thus, they also found that the bovine was stronger and the avian weaker than the human type protein

Recently Aronson, Parr and Saylor (1) have reported intracutaneous cross-reactions in subjects who were highly sensitive to the human purified protein derivative, with 0 00002 mgm doses of protein fractions (TPTs) of *Mycobacterium phlei*, *M smegmatis*, *M ranae*, *M marinum*, *M avium*, *M butyricum*, *M leprae* and *M thamnopheos* There was also evidence of regional variation in the sensitizations For example, in the Philadelphia and Arizona areas, there were 18 2 and 17 1 per cent reactors to the *M phlei* protein fraction, in contrast to 0 4 per cent in the South Dakota area In the same areas, 98, 82 and 54 per cent respectively of those tested reacted to the human purified protein derivative tuberculin These reactions cannot be considered non-specific, since about 93 per cent of children vaccinated (2) intracutaneously with BCG vaccine became sensitive and reacted even after two or three years to this purified protein derivative tuberculin

CONCLUSION

Among the proteins of the acid-fast bacilli, none has received as much study as that from the human type tubercle bacillus Most of these studies have been the result of a search for the specific active principle of tuberculin The conclusion that this active substance is protein, has been supported by comprehensive

and convincing evidence, and the apparently divergent views previously held that it was associated with mucoprotein, nucleoprotein, proteose, etc are explained by the existence of a complex interrelationship between the protein, nucleic acid, and polysaccharide molecules in tuberculin Recent physico-chemical technics have demonstrated a fact frequently suspected, namely that tuberculin proteins of various molecular sizes (10,000 to 32,000) are potent Thus, the disagreement in the literature as to whether or not the active material is dialyzable has been due to the fact that different investigators were working with potent molecules of different sizes

Apparently, the biological potency must be inherent in some small, relatively stable part of the protein molecule, and many changes can be effected in the molecule as a whole before there is recognizable loss in the biological activity Tuberculin in its original form, as produced by the tubercle bacillus may, therefore, be considered a reversibly dissociable component system, which consists of active protein, inactive protein, polysaccharide and nucleic acid The relative proportions of these various components and properties (potency, specificity, toxicity, antigenicity) in any fraction would depend upon the degree of dilution and the reagents used for isolating the fraction With this knowledge, it is apparently possible to isolate molecularly homogeneous substances which would repay careful study along the following lines (a) their precise amino-acid composition, (b) their reactions to specific and highly purified enzymes, and (c) their specific chemical groups responsible for the biological properties which have made tuberculin an interesting and important biological product Heretofore analyses of this character have been of doubtful significance because of the impurity and heterogeneity of the products studied

It has been suggested that the mechanism involved in the denaturation of the protein involves a splitting in the unit molecule and then a polymerization of the split, elongated molecules into long thread-like structures capable of forming gels

From the practical standpoint the chemical studies have led to the isolation of a purified and highly potent protein in large quantity for use as a new official standard for tuberculins This

product has been designated "Purified Protein Derivative Tuberculin"

The proteins from several other acid-fast bacilli have been isolated by similar methods, and have been shown mainly by immunological methods to differ from the protein of the human type tubercle bacillus Recent work indicates that a serological group-specificity exists in the polysaccharides, while a type-specificity is due to the proteins of these bacilli

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THE YEASTS

Genetics, Cytology, Variation, Classification and Identification

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CONTENTS

This review is concerned with the cytology and genetics of yeasts, with their induced and spontaneous variations and mutations, with their classification and identification It is limited to papers appearing during the past decade, save that some older papers have been cited for the sake of continuity or clearness No attempt has been made to review the vast literature on the physiology and biochemistry of the yeasts

For some years there has been maintained an international type culture collection of yeasts at the Technische Hoogeschool in Delft, a section of the Centraalbureau voor Schimmelcultures This collection of cultures from a variety of materials and from

all parts of the world has been carefully and systematically worked over by several persons with the direction of Prof A J Kluyver, and the results are being published as a series of monographs The first appeared in 1931—"Die Sporogenen Hefen" by Fr N M Stelling-Dekker A second division, "Die Anaskosporogenen Hefen" by Frl Jacomina Lodder, is in two portions The first half, dealing with asporogenous yeasts which do not form mycelium or pseudomycelium, was published in 1934 The second half, dealing with asporogenous yeasts which do form mycelium, the *Mycotoruloideae*, has not yet been published This review has largely been built up around these two monographs Partly because the second half of Miss Lodder's monograph is lacking, and partly to keep the review within a reasonable size, the *Mycotoruloideae* will not be reviewed

Any discussion of yeasts must be preceded by a definition of the limits of the group, or hopeless confusion will result Many bacteriologists with little experience in studying yeasts think that they know very precisely what a yeast is, and define it as a unicellular fungus multiplying by budding Actually such a definition will apply only to a small proportion of the organisms usually classified as yeasts, and only to these when they are maintained under constant conditions and not studied too closely Kohl (1908) begins his book, "Die Hefepilze", with the statement that "Die Hefepilze umfassen die Sprosshefen, die Spalthefen und Hefeahnliche Pilze" The "Sprosshefen" are the common unicellular yeasts which multiply by budding, the "Spalthefen" are a series of tropical yeasts, producing alcoholic fermentation and forming multiple endogenous spores like the budding yeasts, but which multiply by binary transverse fission, and the "Hefeahnliche Pilze" are fungi which regularly and obviously produce true mycelium (*i e*, they are not strictly unicellular) but in which a unicellular yeast-like growth form is usually dominant One might with reason exclude this last group, and define yeasts as unicellular fungi, if it were not for the fact that a number of yeasts normally unicellular have been found to give rise to mycelium in certain media, or to give rise to variants that permanently produce mycelium in all media Even the common

industrial yeast, *Saccharomyces cerevisiae*, the type organism about which our conception of the category "yeast" has grown, will often produce a fringe of true mycelium burrowing into the agar at the edge of giant colonies 4 to 6 months old Yeasts, then, are not exclusively unicellular, nor do they multiply exclusively by budding The closest one may come to a satisfactory definition is to state that they are fungi with nuclei in which the usual and dominant growth-form is unicellular

While the central concept of this category is clear, to anyone who will study large numbers of yeasts and yeast-like fungi, it must become obvious that this group of microörganisms shades off by such slight transitions into various groups of higher fungi that they cannot be considered a natural phylogenetic group And while it is equally clear that the bulk of the organisms which we call yeasts are lower Ascomycetes or imperfect forms of these, it is by no means certain that all of them are, there is strong evidence that some of them are Basidiomycetes It is well to keep in mind, therefore, that the term "yeast" does not have the precise botanical significance that such terms as "mushroom" or "moss" or "fern" have

The true relationships of the yeasts must be derived, as with other fungi, from a study of their sexual reproduction Such a study also involves cytology and genetics, which in turn have an important bearing upon the problems of variation that must be considered in any discussion of taxonomy Our knowledge of the cytology and sexual reproduction of the yeasts we owe very largely to Guilliermond, though in recent years important knowledge has been contributed by others, notably Winge and Laustsen It will not be necessary to review all of this work in detail, because this has recently been done by Guilliermond (1940)

GENETICS OF YEASTS

Sexual reproduction in yeasts was first clearly recognized by Guilliermond in 1902 in *Schizosaccharomyces* and by Barker in *Zygosaccharomyces* These observations were amply confirmed, and in addition, heterogamic conjugation between cells differ-

entiated by size was observed in various budding yeasts, notably members of the genera *Debaryomyces* and *Nadsonia* These studies left no doubt that some of the unicellular fungi which we call yeasts exhibit sexual reproduction and that the sexual spores are ascospores

In a large number of yeasts, especially those of industrial importance, however, spores are formed without any trace of conjugation preceding spore-formation, and these yeasts have until very recently been considered to be parthenogenetic, *i e*, devoid of sexuality Since in yeasts which reproduce sexually, Guilliermond had observed some cells forming fusion tubes which failed to fuse, such cells proceeding to form spores parthenogenetically, and in other cases he observed many cells forming fusion-tubes without actual fusion taking place, he postulated that sexuality in yeasts is undergoing a retrograde evolution in which the various steps are Conjugation → Fusion tubes without conjugation → Parthenogenesis One might well extend this concept to include as the last stage the complete loss of the power to form spores The asporogenous or imperfect yeasts would thus represent the end of this evolutionary series

Guilliermond described an additional type of sexuality in *Saccharomycodes Ludwigii* observed by Hansen as early as 1893 This yeast forms usually four spores without previous conjugation On germination, however, the spores conjugate within the mother cell, two by two, so that only two vegetative cells emerge from each ascus containing four spores This process Guilliermond designated parthenogamy, and he considered it as further evidence of degradation in sexuality, placing this process just ahead of parthenogenesis in the retrograde evolution

Guilliermond's observations and theories have been accepted generally by those working with yeasts Beginning in 1935, however, a series of papers by Winge, and by Winge and Laustsen have extended greatly our knowledge of sexuality in the yeasts, and have presented facts of wide significance and importance Briefly, they have shown that the process which Guilliermond called parthenogamy (*i e*, conjugation of spores, or of cells derived from spores) occurs in the industrial yeasts of the genus *Sac-*

charomyces, as well as in *Saccharomycodes*, and that it is doubtful that any of the spore-forming yeasts are entirely parthenogenetic The vegetative cells of such yeasts are normally diploid, but haploid cells may be derived from single spores, and such haploid cells from different races may be caused to fuse and give rise to hybrids

Winge (1935) described the germination of spores of *Saccharomyces cerevisiae* (race Johannisberg II, varieties *ellipsoideus* and *marchalianus*) and of *Saccharomyces validus* In all of them spores were found to fuse by pairs on germination, but not all of the spores fused Spores which germinated without fusion gave rise to small cells tending toward a globular form, whereas fused spores produced larger elongated cells The small globular cells were recognized as haploid, the larger elongated ones as diploid Haploid vegetative cells derived from a single spore often fuse with other haploid vegetative cells to produce a large diploid cell Cytological studies showed that the fusion of spores or of haploid vegetative cells was followed by nuclear fusion "A striking feature is the absence of an established system governing the zygote formation Sometimes two spores unite to form a zygote before or after they have germinated singly, sometimes a spore unites with a haploid vegetative cell, or two vegetative cells conjugate, and they may be sister-cells, or mother-cell and daughter-cell, or more distantly related cells "

These observations indicate that with regard to sexual behavior, yeasts may be divided into two great classes In one (the genera *Schizosaccharomyces, Zygosaccharomyces, Zygopichia, Debaryomyces, Nadsonia, Nematospora*) the vegetative cells are *haploid* The diploid phase extends only through the short period following nuclear fusion This is immediately followed by meiosis, the spores being haploid, and giving rise to haploid vegetative cells on germination In the other group (*Saccharomyces, Saccharomycodes, Hansenula*) the vegetative cells are *diploid* Meiosis occurs during spore formation, and the spores are haploid These may give rise to haploid vegetative cells, but the latter are small and not vigorous The spores, or vegetative cells derived from them, fuse to give rise to the diploid form producing large

and vigorous cells. Guilliermond (1940) applied the term "haplobiontic" to those yeasts whose vegetative cells are normally haploid, and "diplobiontic" to those whose vegetative cells are normally diploid, but a note by B. O. Dodge appended to Guilliermond's article points out that these terms have been used in a different sense.

Winge and Laustsen (1937) developed a micromanipulative technique by which the four spores of an ascus could be separated, isolated, and studied in pure form. Working with a culture derived from a single cell isolated from a strain of *Saccharomyces cerevisiae* used in commercial pressed yeast manufacture, they found that sometimes a single spore gave rise to a pure haploid culture, but in other cases the cells derived from the single spore were diploid from the beginning. They explain the latter cases by assuming that an intracellular nuclear fusion takes place immediately following the first division of the spore nucleus. Diploid cells derived from a single spore readily formed spores when transferred to a suitable substrate. Haploid cells derived from a single spore either failed to form spores at all, or did so only after fusion had taken place between two vegetative cells. Thus a haploid race derived from a single spore may behave either like *Zygosaccharomyces*, i. e., a yeast of the type which Guilliermond designates "haplobiontic", or like an asporogenous yeast of the genus *Torulopsis*. Since yeasts of the latter type are usually small globular forms, it was natural to assume that such yeasts might be haploid and self-incompatible, and that if they were brought into cultures of other races or species, diploidization and spore formation might occur. Accordingly they tested the species of *Torulopsis* in the Carlsberg collection, but in no case did spore formation occur. Nevertheless, in the opinion of the reviewer, this still remains an attractive field for investigation.

Winge and Laustsen (1937), discussing the occurrence of genetic segregation in the ascus, point out that since the vegetative cells of *Saccharomyces* species are normally diploid, a single-cell culture of one of these yeasts will not be necessarily a "pure" culture, since in spore formation segregation of new types may occur,

and spore formation may be easily overlooked That such segregations actually occur was observed repeatedly when giant colonies were started from each of the four spores of an ascus These differed in contour and texture The parent culture was therefore heterozygous That cultures from isolated spores were homozygous was shown by producing new giant colonies from spores produced from a single spore culture These "bred true" Obviously, to study mutations in these yeasts it would be better to have a single-spore culture than a single-cell culture

A third paper (Winge and Laustsen, 1938) is not available to the reviewer but has been abstracted briefly in the fourth paper (1939a) The authors state that they showed "how hybridization of different yeasts is arranged by placing in a droplet of culture solution two (haploid) spores of different species so as to enable them to copulate, forming a (diploid) zygote from which the hybrid yeast germinates" Moreover, this procedure now makes it possible to undertake rational breeding work with yeasts, and possibly to produce new yeasts of commercial value

Winge and Laustsen (1939a) then proceeded with such breeding experiments Single spores were isolated from several strains of *Saccharomyces cerevisiae*, and from *S italicus*, *S validus*, *S mandshuricus* and *Zygosaccharomyces priorianus* These spores were brought together in pairs and when conjugation had been observed to take place, subcultures were made and various characters, particularly giant colonies, were observed Giant colonies of 14 new hybrids differed in texture and contour from both of the parent types The hybrids were also compared with the parental types as regards their ability to ferment sugars In all cases, ability to ferment was a dominant character, if one of the parent types fermented sucrose, for instance, and the other not, the hybrid invariably fermented sucrose

Crosses were obtained not only between different races of *Saccharomyces cerevisiae*, but also between different species of *Saccharomyces*, and in one instance an intergeneric hybrid (*Zygosaccharomyces priorianus* x *Saccharomyces cerevisiae* Rasse II) was obtained The relation of such inter-fertility to problems of taxonomy is discussed When the spores of the parental

types were tested, 43 to 90 per cent of them were capable of germination Spores derived from hybrids fell into two groups, one showing germination of 0 to 13 per cent of the spores, the other 50 to 94 per cent The latter are considered to be examples of intraspecific hybrids, the former of interspecific hybrids Hybrids between two species may be propagated by budding, but tend toward sterility in sexual reproduction This provides a method for testing specific relationships In all cases where the parent types differed in fermentation characters, the hybrids showed reduced germination of the spores Strains showing the same fermentation characters are not necessarily specifically identical, one cross between two yeasts giving identical fermentations yielded hybrids with very poor spore germination

The use of these facts in breeding and selecting better industrial yeasts is pointed out by the authors They found that a typical top yeast may segregate out a typical bottom yeast through its spores, and concluded that there is no definite line of demarcation between these two types of brewing yeasts One of their hybrids proved to be superior in compressed yeast manufacture

Saccharomycodes Ludwigii is an interesting yeast whose four spores occur in two pairs, often the two spores in each pair attached, and whose spores fuse in germination Winge and Laustsen (1939b) reported on genetic studies with this yeast When the four spores are isolated, and forced to germinate without fusing, they show differences in growth Some spores yield small, globular buds, others long cylindrical ones In some cases growth continues normally, in others growth ceases after a very small number of buds have formed These characters depend upon the Mendelian segregation of two pairs of genes Gene *N* gives rise to normal growth, while *n* causes a cessation after a single short hypha has formed, gene *L* produces long cylindrical cells, *l* short globular ones These characters may be segregated as *Nl* and *nL*, and this is a matter of chance But in each pair of spores in an ascus, one has one set of genes and the other the other, either *Nl* and *nL*, or *NL* and *nl*

If the isolated spore is capable of growth, (*i e*, if it contains *N* and not *n*) it will grow continuously as a haploid yeast, it never

diploidizes and therefore never forms spores. Spores having the formula *NL* give rise to elongated cells which form giant colonies "characteristically scaly or rather lobed, under the lens tufty." Spores having the formula *Nl* give rise to smooth colonies. Colonies derived from diploid cells (*Nn Ll*) are characteristically rough. Consequently the rough or lobed colony character is dominant. The authors also obtained a mutant which produced a new race having the formula *NNLl*, in which each of the four spores gives rise to continuous growth.

CYTOLOGY OF YEASTS

The researches of Winge and Laustsen indicate that yeasts behave like higher organisms with regard to the transmission of hereditary characters in sexual reproduction, and this observation naturally gives rise to renewed interest in the cytology of yeasts, especially in the behavior of the nucleus during vegetative and sexual reproduction. Bacteriologists have been interested in the nuclei of yeasts because these microbes have seemed to be the next step above the bacteria in scale of size and complexity, and it has seemed that methods which would certainly demonstrate nuclei in yeasts might be applicable to bacteria. Yeast cells are usually packed with a variety of stainable granules or globules, and earlier studies did not distinguish these clearly. It is probable that Moeller in 1893 saw and photographed the true nucleus of yeasts, but the certain existence of a nucleus was not definitely established until the researches of Guilliermond in 1901. Earlier literature on the nuclei of yeasts has been reviewed extensively by Kohl (1908) and Guilliermond (1920).

Yeasts, especially *Saccharomyces cerevisiae*, are rich in volutin partly deposited in granules in the cytoplasm and partly dissolved in the fluid of the vacuoles. Early workers did not always distinguish this material from the chromatin of the nucleus, since both stain with the basic dyes. In particular Wager (1898), one of whose drawings has been widely reproduced in textbooks, mistook the vacuole and its granules for the nucleus, as was pointed out by Guilliermond (1920). Although means for differentiating volutin from chromatin (vital staining with neutral red, solubility

in hot water) were available earlier, the development of the Feulgen method for the microchemical demonstration of thymonucleic acid *in situ* has provided a more certain method for this differentiation The application of the Feulgen method has demonstrated the occurrence of thymonucleic acid in the nuclei of yeasts, and it is now certain that yeasts produce two nucleic acids "yeast" nucleic acid, abundant, probably identical with the volutin granules, and thymonucleic acid, scant, contained in the nucleus

Though Feulgen failed to obtain a positive "nuklealreaktion" in yeasts, Margolena (1932), and Pietschmann and Rippel (1932) obtained positive reactions which were, however, so faint that a satisfactory microscopic picture was not obtained Imšenecki (1936) obtained a positive reaction with masses of yeast cells, but could not see the color in the individual yeast cells Rochlin (1933) on the other hand, obtained a very definite picture with *Saccharomyces cerevisiae* The Feulgen method gave an intense and beautiful staining of the nucleus, no other structure was colored The nucleus, 0 8 to 1 3 μ, was vesicular, with the chromatin arranged as a crescent along one side A similar picture, with less shrinkage, was observed in slides stained with iron hematoxylin Winge (1935) states that Laustsen also developed a modification of the Feulgen method which gave a better differentiation of the nuclei than is obtained with hematoxylin Badian (1937) used Rochlin's technique, and obtained identical results, which also checked the results of his modified Giemsa stain

Although it has long been certain that yeasts show single, discrete nuclei, it is still not certain how these behave during budding or fission, conjugation and spore formation These nuclei are about 1 μ in diameter, and in attempting to see what goes on in such a small body there is always a temptation to strain the resolving power of the microscope to the utmost It is my opinion that descriptions of details in morphologic structures less than 1 μ in diameter should always be taken *cum grano salis* It is to be hoped that the development of the electronic microscope will solve some of these riddles

Earlier investigators were about evenly divided between those

who saw only amitotic division of the nucleus, and those who pretended to observe the formation of chromosomes and division by karyokinesis, at least during spore formation. This work has been reviewed by Badian (1937). Guilliermond maintained until recently that division during budding is amitotic, but in 1917 described a sort of mitosis in *Schizosaccharomyces octosporus*. This yeast produces 4 or 8 ascospores following conjugation. The nucleus of the zygote is large and vesicular, containing a nucleolus and several granules of chromatin. On division, an achromatic spindle is formed within the nuclear membrane with the chromatin granules (very small) gathered as an equatorial plate in the center. The nuclear membrane is absorbed, the chromatin granules move toward the two poles, the spindle elongates, and the chromatin at the two poles gathers together to form the two daughter nuclei. The nucleolus, left in the middle of the cell, disappears. Second and third divisions take place in a similar manner, the daughter nuclei dividing simultaneously. The chromatin granules, considered to be chromosomes, are too small to count. The entire process is very difficult to demonstrate due to the small size of the nucleus. Guilliermond (1940) stated that he observed mitosis-like features during budding in *Saccharomyces paradoxus*.

Kater (1927) ascribed the widespread acceptance of amitotic division as the normal process in budding to the difficulties encountered in attempting to study nuclear division in such small organisms, and pointed out that the development of genetics makes it now impossible to accept amitotic division as a normal process. He studied *Saccharomyces cerevisiae* by fixing wet smears in Bouin's fluid. If these are stained in iron hematoxylin before all of the picric acid has been washed out, the nuclear structures are revealed without interference from the metachromatic material. The resting nucleus contains a nucleolus, with chromatin as granules at the periphery. In budding, this chromatin becomes arranged upon linin strands that form an achromatic spindle, and becomes divided into chromosomes, probably at least 8 in number. Both the resting nucleus and the mitotic figures resemble those of *Phaseolus*. There is no description of the nucleus in sporulation.

Badian approached the study of nuclear phenomena in yeasts

following a series of investigations, dating from 1930, on nuclei in bacteria, myxobacteria, and actinomycetes. In these primitive organisms he claimed to observe free in the protoplasm rod-shaped chromosomes which divide longitudinally at cell division. Fertilization occurs by autogamy, the chromosomes fusing to form bivalent chromosomes. A return to the haploid phase is accomplished by a reduction of the chromatin, part of the chromosomes disappearing after two successive divisions of the bivalent chromosome. These papers are quoted by Badian (1937).

Badian studied the nuclei of *Schizosaccharomyces pombé*, *Saccharomyces cerevisiae*, *Saccharomycodes Ludwigii*, and *Rhodotorula glutinis*. Wet smears were fixed in osmic acid vapor and stained by a modified Giemsa method, or by the Feulgen method according to Rochlin. In *Schizosaccharomyces pombé*, the nucleus of the resting cells is globular with the chromatin arranged as a crescent at one side. In fission, this chromatin is arranged as two rod-shaped chromosomes, which divide transversely to form four, two moving to each pole of the cell, after this a cross-wall is formed, and the two chromosomes in each cell fuse to form a globular nucleus. In fertilization, the chromatin in each of the cells about to fuse also appears as two rod-shaped chromosomes. Upon fusion their chromosomes join at the ends, to form two long (bivalent) chromosomes. These become shorter and fatter, and occupy a position in the center of the cell at right angles to its long axis. These now divide longitudinally to form four, and one pair passes to each end of the zygote, where they divide again (transversely) to form now four pairs of chromosomes, each pair becoming a spore nucleus. In some cases some of the spore nuclei degenerate, so that the number of spores may be less than four.

In *S. cerevisiae*, the resting nucleus appears the same as in the preceding yeast, and forms two chromosomes. The division of the nucleus may precede the appearance of a bud, and takes place in the middle of the mother-cell. The two chromosomes divide longitudinally and one pair passes to the bud where they become transformed into a new resting nucleus. In spore formation, the pair of chromosomes undergoes two divisions, yielding four pairs of chromosomes, each pair forming a spore nucleus. Again, some

chromosomes may degenerate without forming spores. In spore germination, the spores fuse and immediately the zygote commences to bud. In the fused spores the two pairs of chromosomes fuse end-to-end, then divide longitudinally, one of the two pairs thus formed passing into the bud.

The vegetative division of the nuclei in *Saccharomycodes Ludwigii* and *Rhodotorula glutinis* proceeds in the same manner as in *S. cerevisiae*.

Badian points out the similarity between nuclear division as he observed it in the yeasts, and the same process observed in higher fungi, and also as he observed it in bacteria. It is difficult to evaluate Badian's work without repeating it, and it involves a delicate and painstaking staining method. One's first reaction is that he has seen more than the microscope will reveal in such minute bodies as bacteria and yeasts. Guilliermond (1940) states that "His figures, however, are too diagrammatic to be taken into consideration; they fit neither with our nor with his own photomicrographs." This criticism is largely true, though several of Badian's photomicrographs clearly show paired bodies similar to the chromosomes he describes. At any rate, Badian's observations are so striking and important that they deserve serious consideration and careful repetition. Certainly the two chromosomes described by him are more definite than the vague accumulations of minute chromatin granules described by Guilliermond. The occurrence of bivalent chromosomes rather than a doubling of the chromosomes in the diploid phase is noteworthy.

Renaud (1938), with *Saccharomyces ellipsoideus* fixed in Bouin's fluid and stained with iron hematoxylin, reported that in budding, a centrosome appears on the surface of the nucleus facing the bud. This divides, and one part enters the bud. The membrane dissolves, an achromatic spindle is formed, and the chromatin becomes arranged in chromosomes that form two masses in the middle of the spindle. The number of chromosomes could not be counted because of their small size, but Renaud believes that there are more than four. He states that mitosis is similar to that observed in higher Basidiomycetes, and much more complex than the process described by Badian.

Richards (1938) attacked the problem of mitosis in yeasts by

the use of colchicine, which is known to arrest mitosis in the metaphase with higher organisms He believed that if yeast nuclei divide by mitosis, the addition of colchicine to the medium should retard growth, and the chances of finding cells in mitosis would be increased Instead, colchicine stimulated growth and no chromosomes were found in preparations stained with iron hematoxylin, by the Feulgen method, or examined by ultra-violet microscopy

Winge and Laustsen have not described in any detail cytologic studies to accompany the genetic studies which they have published, though they promise such a paper in the future Winge (1935) described and illustrated unequivocally the nuclei and their fusion in the zygotes of *Saccharomyces ellipsoideus* (strain Johannisberg II) In the higher Ascomycetes and Basidiomycetes a characteristic feature is a delay between cell fusion and nuclear fusion, the zygote giving rise to binucleate mycelium Guilliermond (1940) claims that yeasts show a tendency in this direction In *S paradoxus*, the nuclei of the zygote may fail to fuse, but show "conjugate division," the two nuclei dividing simultaneously to provide two nuclei for the first bud, in which they fuse Renaud (1938) observed the same thing in a strain of *S ellipsoideus*, two or three buddings with conjugate mitosis preceding fusion of the nucleus

We may summarize the newer developments in the cytology of yeasts by stating that there can no longer remain any doubt that they possess single discrete nuclei There can remain a legitimate doubt regarding the method of nuclear division, but the balance of evidence favors the conclusion that both in budding and spore formation the nucleus divides by mitosis as in higher organisms This is supported especially by the papers of Winge and Laustsen which show that in genetic behavior the yeasts behave precisely like higher organisms, and must depend, therefore, upon a chromosomal apparatus In one case (Winge and Laustsen, 1939b) they were able to show by segregations in the spores of *Saccharomycodes Ludwigii* in which planes the nuclei divided, and in which divisions the characters were segregated

VARIATIONS IN YEASTS

In addition to the investigations of Winge and Laustsen on the genetics of yeasts, there have appeared during the last decade a number of papers on the occurrence of spontaneous and induced variations in yeasts and yeast-like fungi Scattered earlier observations had indicated that such variations do occur, particularly the development of mycelial races from normally unicellular yeasts, and of asporogenous races from normally spore-forming yeasts Much of the more recent work has been done by Nadson and his associates in Leningrad, and since many of the publications are in Russian, they have been available to me only as abstracts and quotations While some purely spontaneous variations have been observed, most of the observations have concerned variations induced by exposure of the yeasts to injurious agents,—chloroform, cyanide, lithium chloride, immune serum, x-rays, and radium emanation Some of the variants have been relatively stable for a time, but have eventually reverted, these Nadson designates "dauermodifikationen" Others have remained permanent, and these he designates as "saltants" It is noteworthy that Nadson recorded the production of variants in lower fungi under the influence of x-rays previous to the publication of Muller's fruitful researches upon x-ray mutants of *Drosophila*

Nadson and Philippov (1928) exposed a "rose yeast" to x-rays Subcultures plated from the exposed cells showed sector mutants in the colonies In the original culture the colonies were smooth to mucoid, the cells round, encapsulated, and filled with fat A variant was obtained which showed the same color, but the growth was dry, the surface matted and folded The cells were cylindrical with little oil and no capsules, and they reproduced by exogenous spores borne upon sterigmata, precisely as in the yeast *Sporobolomyces roseus*, previously described by Kluyver and van Niel A second variant was similar, but the color was light orange rather than rose These variants remained stable over three years (80 culture generations) and could not be caused to revert by renewed exposure to x-rays They note also the

development of asporogenous races from the sporogenous yeast *Nadsonia fulvescens* under the influence of x-rays

Derx (1930) observed spontaneous variations in yeasts of this same group When freshly isolated from nature (leaves of plants) species of *Sporobolomyces* grow as pink mucilaginous colonies On these colonies, firm rough secondary colonies may appear The mucilaginous colonies show only budding globular or oval cells, the secondary colonies show elongated cells or mycelium, and form the characteristic sporobolomyces type of spore, which give the surface of the colony its powdery appearance By repeated re-inoculation of these spores there may be obtained a race purely rough and sporulating, without any mucilaginous stage The same transformation was observed in liquid media, the rough sporulating type appearing as a pellicle If cultures are transferred at frequent intervals using the vegetative cells rather than spores, a reversion of the rough form to the mucilaginous type may be obtained Derx also records another type of variation in *Sporobolomyces*, a sudden appearance of a single white colony among many (normal) pigmented ones This mutant was identical with the parent type in all characters save for the lack of pigment, and the variation was permanent

Nadson and Krassilnikov (1932) recorded spontaneous variations which occurred over a period of three years in a yeast-like fungus, *Guilliermondella selenospora*, belonging to the *Endomycetaceae* Five races emerged One was characterized by abundant typical mycelium and parthenogenetic asci A second showed poorly developed mycelium and many yeast-like cells, with spore formation preceded by conjugation A third showed prickly mycelium and no spores A fourth yielded colonies with tufts of mycelium on the surface and showed no spores The fifth was entirely yeast-like with no mycelium, and showed conjugation between the bud and mother-cell This last race also differed from the parent fungus by its ability to ferment mannose, glucose, and fructose

Philippov (1932) studied the influence of x-rays upon the red yeast, *Rhodotorula glutinis* New races were obtained which differed in the fat content of the cells, the character of the budding, the production of gum, and pigmentation

Punkari and Henrici (1933) described spontaneous variations in an asporogenous yeast, *Torulopsis pulcherrima*. This was an old laboratory culture originally isolated by Grosbusch, which had become unstable. Variants appeared as sectors or as secondary colonies in giant colonies, and resulted from aging of the colonies, or spontaneously. Variations in color (red and white) and in texture (smooth and rough) were observed to occur independently. Roughness was associated with the development of mycelium in both red and white variants. The authors emphasized that these variations occurred in a yeast which had never formed spores, and compared them in significance to the variations occurring in haploid cultures of smut sporidia described by Christensen. The later researches of Winge and Laustsen support the view that asporogenous yeasts may be haploid and self-infertile. Such variations then must be regarded as mutational and not the result of segregation or of hybridization. In a second paper (Punkari and Henrici, 1935) further observations of the same yeast are recorded. It was noted that white variants from the original red yeast are much more stable than the parent stock, that white variants arise from red usually as sectors, *i.e.*, at the edge of the growing colony, while in those rare cases where white reverted to red, this occurred as secondary colonies in the center of the colony. The contention that the variations were independent of any sexual process is not certain in view of the later observation by Windisch (1938) that *Torulopsis pulcherrima* does form ascospores by isogamous conjugation. Spore formation occurred in the presence of a species of *Penicillium*, and in very old agar cultures dried to such a degree that the growth adhered to the agar.

Nadson and Rochlin (1933) reported upon variations induced by exposing cells of *S. cerevisiae* to radium emanation (radon). This was applied in glass capillaries in doses of 19.6 to 23.3 millicuries, placed in bottles containing 10 ml. of beer wort. The capillary was then crushed, and the liquid inoculated with the yeast. After 24 to 48 hours the cultures were plated on wort agar, and the resulting colonies compared with control colonies from cultures in wort without radon. From the normally smooth type parent strain, two rough variants were obtained, one with

small globular cells, the other with large cylindrical cells, resembling species of *Torulopsis* and of *Mycoderma* respectively Another variant was characterized by abundant spore formation, producing spores readily in beer-wort, which the parent strain would not do On re-exposing this sporulating variant to the radon, a new type with restricted rugose colonies and distorted cells was obtained The sporulating race is considered a "dauermodifikation," but the other three variants, irreversible in many generations, are considered "saltants," the equivalent of "mutants" in higher organisms These new races differed quantitatively in ability to ferment sugars, and in volume of crop One was an improvement over the parent strain in producing more alcohol, a larger crop, and in settling more quickly

Meissel (1933) reported on variants of *S cerevisiae* induced by the action of potassium cyanide The yeast is relatively resistant to this poison, surviving an exposure of 24 hours to a 5 per cent solution Plates made from suspensions so treated yielded a great variety of variants, both morphological and physiological, 40 in all, some of which reverted after a time, others appeared to be permanent Rugose colonies in some cases showed globular cells, in other cases elongated cells Variants re-exposed to KCN underwent renewed variations A noteworthy feature was the development of variants which, after growing for a time, underwent spontaneous autolysis accompanied by a dark brown discoloration of the colony From these autolyzed areas secondary colonies sometimes grew, which showed new variations These autolytic variants may be related to the lethal variants noted by later authors

Krassilnikov (1934) observed spontaneous variations in *S cerevisiae* These developed in old (1½ to 2 months) cultures Some 25 new races were obtained, divided into five groups differing from the parent culture in stability, in cell form, in colony form, and in physiological characters A noteworthy variant was a "lethal" form, with brown colonies and reduced resistance to unfavorable influences, a tendency to die if not frequently transplanted Variants also appeared in 10 to 12 days in cultures grown at high (37°) temperature, appearing either as sectors or

as secondary colonies. These resembled the variants obtained spontaneously by growth at a lower temperature over a longer period of time.

Rochlina (1934) obtained variants of *S. cerevisiae* through the action of radium. Wort agar was thickly seeded with the yeast, and poured into Petri plates with a capillary tube of radium in the center. Immediately about the tube there was no growth, but in the inhibitory zone, some colonies appeared which were variants. Among these was a stable asporogenous race with ameboid cells, cells with numerous projections which are considered to be undeveloped buds with thin walls. Colonies were rough and cells showed cytological differences from the parent type.

Fabian and McCullough (1934) reported on induced variations in yeasts; in this report, obviously, they were influenced greatly by the work of Hadley and others on dissociation in bacteria. They worked with *S. cerevisiae* (strains Saaz and Frohberg), *Hansenula anomala*, and *Zygosaccharomyces mandshuricus*. Variations induced by aging or by serial transfers in broth containing lithium chloride, broth containing brilliant green and picric acid, broth containing alcohol, or by desiccation on gypsum blocks or by growth at different temperatures produced three main types: the *S* or smooth form, with globular cells and smooth colonies; the *R* or rough form with elongated cells and rugose colonies; and the *G* or gonidial form, with small bacteria-like colonies. Growth in lithium chloride and brilliant green broths gave rise to both *R* and *G* forms. Desiccation on gypsum blocks gave rise to *G* forms but no *R* forms. The production of *G* forms was stimulated by growth in broth containing alcohol.

R forms in general showed the same biochemical characters as the *S* forms from which they were derived, but produced pellicles on liquid media. The *G* forms, however, differed markedly, failing entirely to produce alcoholic fermentation, but producing acid without gas from some sugars.

R and *G* forms reverted to the *S* forms on repeated transfers in malt extract broth. *G* forms were said to arise from both *S* and *R* forms, either by a gradual shrinkage in the size of the cells,

or suddenly by the production of multiple small buds A transitional, or *T* form, is also described as preceding the appearance of *G* forms The reversion of *G* forms to *S* occurred suddenly without any transitions

The *G* forms described and illustrated by Fabian and McCullough are obviously bacteria, and it is difficult for the reviewer not to believe that they are bacteria which have contaminated the cultures rather than offspring of the yeasts There is further evidence of contamination in experiments reported by these authors Thus the *G* forms of the Saaz yeast are described as changing to a pink mucoid yeast, obviously *Rhodotorula glutinis*, and to an *R* form with black wrinkled colonies, obviously *Monilia nigra*, two of the common air contaminants which appear frequently on yeast media Though the uncritical character of the work makes it difficult to evaluate, the *R* forms described have been observed repeatedly by others

The experiments of Nadson and his coworkers were continued with *Zygosaccharomyces mandshuricus* by Olenov (1935) He retained the radon in capillary tubes which were placed upon the surface of agar plates seeded with the yeast, only the beta and gamma rays were used After 1 to 4 days the radon tube was removed Each colony which developed was examined microscopically and grossly, and an approximately equal number of control colonies (not exposed to radon) were similarly examined This yeast proved more stable under radon treatment than did the one studied by Nadson and Rochlin, and single exposures gave rise to only slight variations By exposing such variants again to radon, new variants were produced, and after 4 such re-exposures, 15 new races were obtained It is noted that this amount of exposure would be lethal if continuous In three instances new variants developed spontaneously in subcultures from races previously exposed to radon, but no variants developed spontaneously in the parent strain which had never been exposed to radon The variants differed from the parent race in many characters which varied independently The parent type gave smooth giant colonies, while giant colonies of most of the variants were rough Cell forms varied from globular to

cylindrical, in one case irregular or ameba-like cells While spore formation in the parent race was preceded by isogamous conjugation, in some of the variants it was parthenogenetic, and others were asporogenous. Though the parent strain fermented only glucose, twelve of the variants fermented galactose, sucrose and maltose as well

In further studies (Olenov, 1936) it was shown that one of the induced variants, asporogenous and capable of fermenting maltose, was more successful than, and would crowd out, the parent race in beer wort This was attributed to the ability of the new race to ferment maltose, but it was found capable of overgrowing the parent race in other media Thus the author claims to have carried out a degree of experimental evolution, involving the development of new races by induced mutation, the adaptation of one of these to, and its selection by, the environment (beer wort)

Wickerham and Fabian (1936) described differences in the morphology of the *S* forms, and of *R* forms induced by cultivation in media containing lithium chloride They worked with *Saccharomyces aceris-sacchari* Fabian and Hall, and with *Pichia alcoholophila* The cells of the *R* forms were characterized by elongated form, abundance of large oil drops and larger vacuoles Both cells and colonies were larger than in the *S* forms In cultures of the *R* form, daughter cells separate immediately, while they tend to remain attached to the mother cells in the *S* cultures The *R* cells tend to float to the top of the liquid media, *S* cells tend to sink

Nadson (1937) presented generalizations drawn from the previous papers by himself and his students It is noted that the effect of x-rays is not apparent immediately, and that variations may not appear until subcultures of the exposed culture are studied Induced variations may or may not be hereditarily transmissible But it is noteworthy that temporary modifications are of the same character as permanent ones, and whether they become hereditary or not depends largely upon the dosage of the inciting agent Certain observed variations are considered to be atavistic in character, for example the development of the

spore-forming *Sporobolomyces* from *Torulopsis* types No incontestably progressive or evolutionary changes have been observed The age of the culture treated is of importance, young and old cultures are more likely to give rise to variants when exposed to radiation than are cultures of middle age, and old cultures are more likely to form variants than young ones There is an optimal dosage of the inciting agent Both radiation and chemical agents incite the development of variations of the same character According to dosage such agents produce effects which may be indicated by the range No effect → Stimulation → Depression → Necrobiosis → Death Variations may appear in cells exposed to the range, Stimulation → Necrobiosis, which Nadson calls the "field of action" From a single strain of *S cerevisiae*, stable races were obtained which corresponded in morphology with seven other species The review concludes with a complete bibliography of the papers from Nadson's laboratory, only some of which have been cited here

Throughout this series of studies on genetics and variation of the yeasts there runs a common thread Whether arising by genetic segregation or by hybridization, whether by spontaneous or induced mutation, or by unknown mechanism, there is a tendency for the normally globular yeast with smooth colonies to give rise to forms with cylindrical or sausage-shaped cells, then to pseudo-mycelium, and finally to true mycelium, accompanied by increasing roughness of the colonies Other variations are noted,—the loss of pigment, the "lethal" variants, and losses in fermentative powers and spore formation noted by various workers—but these characters all vary independently of the *S-R* transformation The *S-R* variation in yeasts and yeast-like fungi shows such a close parallelism to this type of variation in bacteria that one can hardly escape the conclusion that they are identical phenomena As with the bacteria, the formation of rough variants is prone to take place spontaneously in old laboratory strains, and may be induced by such agents as lithium chloride, immune serum, x-rays, and radium emanation The rough variants of both groups of microbes are characterized by the tendency to form pellicles on broth, to spontaneous agglutina-

tion or nonspecific agglutination by various agents, to a loss of pathogenicity But there are also morphological parallels The tendency of the cells of the rough forms of yeasts to become sausage-shaped has its parallel in the bacillary forms observed in the rough forms of the pneumococcus, for instance, or in the long filamentous cells found in rough colonies of the gram-negative bacilli The transformation of yeasts to mycelial fungi has its counterpart in the observation recorded by Novak and Henrici (1933) of a coccus transformed into branched mycelium characteristic of actinomycetes One may find in earlier literature, notably in the work of Lieske (1921), a suggestion that unicellular bacteria bear a relation to the actinomycetes similar to that of the yeasts to the molds This is as yet rather vague and conjectural, but it is obvious that it behooves the bacteriologist interested in problems of cytology, genetics and variation to follow the development of these fields in connection with the yeasts and lower fungi

PHYLOGENY OF YEASTS

As early as 1897 Schröter had pointed out the similarity between the spore-forming yeasts and mycelial fungi of the family *Endomycetaceae* Following his earlier studies of sexuality in yeasts, Guilliermond in 1909 began to trace the origin of the yeasts in the higher fungi, and subscribed to the idea that they were derived from the *Endomycetaceae*, a family of primitive Ascomycetes which produce mycelium that may give rise to yeast-like cells by budding or by fragmentation of the mycelium into oidia, and which produce ascospores by isogamous conjugation of contiguous cells in the mycelium In the genus *Endomycopsis* yeast-like cells are formed by budding, and it is a simple transition from such a fungus to budding yeasts of the genus *Zygosaccharomyces*, which form ascospores by isogamous conjugation but produce no mycelium In the genus *Endomyces* free cells are produced as oidia which multiply by fission, and again it is a simple transition from such fungi to yeasts of the genus *Schizosaccharomyces*

The relationship of the ascospore-forming yeasts to the *Endomycetaceae* is so obvious that it has been accepted by all

mycologists, and the spore-forming yeasts have been generally classified as a family, *Saccharomycetaceae*, placed next to the *Endomycetaceae* in the order *Plectascales*, the two families differing only in the formation of mycelium But we have already seen how easy is the transformation from a unicellular yeast-type to a filamentous mold-type Stelling-Dekker (1931) points out how difficult it often is to determine whether a given species is sufficiently mycelial to be placed in the *Endomycetaceae* rather than the *Saccharomycetaceae*, and that the same species has been placed in both families by different authors She considers that a separation of the families is unwarranted, and includes the ascospore-forming yeasts in the family *Endomycetaceae*, of the order *Plectascales* of the Ascomycetes

In his earlier writings Guilliermond derived all of the yeasts from the *Endomycetaceae* through the process of retrograde evolution in sexuality With further development of knowledge of sexuality in those yeasts whose spores conjugate on germination ("diplobiontic") or whose sexuality is manifested by a conjugation of haploid buds derived directly from spores ("haplo-diplobiontic"), Guilliermond has developed a new concept of the phylogeny of yeasts involving two lines of descent, the haplobiontic yeasts which form spores following isogamous or heterogamous conjugation and derived from the *Endomycetaceae* of the order *Plectascales*, and the diplobiontic and haplo-diplobiontic yeasts derived from the *Exoascales*, especially through the genus *Taphrina* This double phylogeny would warrant a sharp separation of the spore-forming yeasts into two groups We will not attempt to review in detail the numerous papers in this field which have appeared from Guilliermond's laboratory since 1931, the following statements being taken from his review (Guilliermond, 1940)

In the *Endomycetaceae*, ascospores are formed by the conjugation of two neighboring cells in the filament of mycelium, which are haploid, the zygote gives rise to ascospores which are diploid These on germination give rise to mycelium, which in turn may give rise to yeast-like cells by budding or by fragmentation The mycelium and the yeast-like cells are haploid In this family

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Guilliermond places the yeasts which he calls haplobiontic (*Schizosaccharomyces, Zygosaccharomyces, Zygopichia, Debaryomyces, Nadsonia, Nematospora*)

In the remaining genera the vegetative cell is diploid, only the spores being haploid in the "diplobiontic" *Saccharomycodes Ludwigii*, or the spores and the first few buds in the "haplodiplobiontic" genera (*Saccharomyces, Hansenula*) These Guilliermond compares to fungi of the *Exoascales*, and particularly to the genus *Taphrina*, the life cycle of which was reported by Wieben (1927) In the parasitic fungi of this genus, the haploid ascospores give rise to haploid budding yeast-cells on germination These soon conjugate, but the nuclei do not at once fuse, instead there is produced a binucleate mycelium, as in higher Ascomycetes and Basidiomycetes Eventually the nuclei fuse to give rise to asci Guilliermond considers this cycle to be similar to that of the diplobiontic yeasts, the diploid phase of the yeasts being represented by the binucleate phase of *Taphrina*, instead of a 2n nucleus, there are two "n-chromosome" nuclei As was mentioned in a preceding section, Guilliermond and Renaud found some tendency toward a binucleate stage in the life histories of some yeasts

Guilliermond's concept of the phylogeny of the yeasts would make the genera *Saccharomyces* and *Zygosaccharomyces* only distantly related, belonging to separate orders of the Ascomycetes Stelling-Dekker's classification makes these two genera but subgenera of a single genus, *Saccharomyces*, in the broader sense In this she is following a suggestion of Klocker She justifies her arrangement on the ground that the *Zygosaccharomyces* species closely parallel the *Saccharomyces* (*sensu strictu*) species in physiological and other characters, that parthenogenesis is common in *Zygosaccharomyces* and conjugation is occasionally seen in yeasts placed in the genus *Saccharomyces* Winge and Laustsen (1937) also point out that "The circumstance that *Saccharomyces* may be cultivated in the haploid state even though it is inclined to revert to the diploid phase by zygote formation shows that there is only a slight biological difference between *Saccharomycetes* in the strict sense of the term and *Zygosaccharomycetes*" The

fact that these authors (1939) were able to obtain hybrids by crossing a species of *Saccharomyces* with a species of *Zygosaccharomyces* indicates that these genera cannot be very distantly related

Guilliermond (1940) further speculates upon a possible relationship between the yeasts and the smut fungi (*Ustilaginales*) The latter, although Basidiomycetes rather than Ascomycetes, show similarities in their life history to the *Exoascales* such as *Taphrina* The smut spore may be considered to be homologous with the asci, containing nuclei which fuse On germination the smut spore produces a short filament of mycelium which gives rise to large numbers of yeast-cells (sporidia) which may multiply as such indefinitely by budding Such smut sporidia, isolated in pure culture, have often been mistaken for true yeasts Guilliermond compares the life cycles of various types of *Ustilaginales* with the various groups of yeasts

There is, however, better evidence that some yeasts are related to Basidiomycetes in a consideration of those belonging to the genus *Sporobolomyces*, the nature of which was first clearly recognized by Kluyver and van Niel (1925) They are yeasts which produce a rose or salmon-colored pigment The colonies become powdery on the surface, and if colonies in this stage are inverted in position, a faint mirror image of the colonies appears on the lid of the Petri dish in which they are contained This image is composed of spores which are forcibly discharged from the surface of the colony The vegatative cells are oval to cylindrical in form and multiply by budding like other yeasts, and a pseudomycelium may be formed Cells on the surface of the colony give rise to fine stalks, or sterigmata, upon which the kidney or sickle-shaped spores are formed When mature these spores are discharged forcibly, the discharge being preceded by the appearance of a droplet of moisture The authors note that the shape, formation and mode of discharge are precisely as Buller had previously described for mushrooms, and considered this evidence that yeasts of this group might be considered to be related to the Basidiomycetes

This was rejected by Lohwag (1926) on the ground that a similar method of spore-discharge is observed in other fungi not related to the Basidiomycetes Lohwag stated that only a cyto-

logical study to determine whether the pretended basidiospores result from a fusion of two nuclei would establish the true nature of the spores Such cytological studies by Guilliermond (1927) revealed but one nucleus in the vegetative cells and the cells which give rise to the spores He concluded, therefore, that the spores are not basidiospores, but asexual conidia This argument was opposed by Kluyver and van Niel (1927) and Buller (1933), who point out that a failure of nuclear fusion does not exclude the possibility that the spores of *Sporobolomyces* are basidiospores because parthenogenetic basidiospores are known in higher Basidiomycetes, and because the ascospores of many yeasts are known to be parthenogenetic in origin Buller, who had studied so carefully the mode of spore discharge in the mushrooms, was fully convinced that the spores of *Sporobolomyces* were typical basidiospores, and this position was also supported by Derx

Derx (1930) found the natural habitat of these yeasts to be leaves and straw, and showed that when freshly isolated they give rise to colonies which are smooth and mucoid, spore-formation first appearing in secondary colonies, or in floating islands of growth upon liquid media He discovered a new group of yeasts which discharge their spores, colorless or slightly yellowish, and with globular or ovoid, symmetrical basidiospores, they are discharged by the same mechanism as in *Sporobolomyces* This yeast Derx placed in a new genus, *Bullera* (which, according to Ciferri and Verona (1938), should be spelled *Bulleria*, in order to conform with the International Botanical Rules of Nomenclature)

Ciferri and Redaelli (1935) considered the spores of *Sporobolomyces* to be conidia rather than basidiospores, and placed these yeasts together with *Nectaromyces Reukaufii* in a family of conidia-forming yeasts, the *Nectaromycetaceae* *Nectaromyces Reukaufii* is a peculiar yeast occurring in the nectar of flowers in groups of four cells in the form of a cross or an "aeroplane" In cultures ordinary budding cells without spores are formed Nadson and Krassilnikov (1927) recorded a mutant which gave rise to mycelium that produced conidia On the basis of this single observation, Ciferri and Redaelli proposed to unite this genus with *Sporobolomyces* It seems to the reviewer that this position

is untenable, and that the genus *Sporobolomyces* represents a group of yeasts derived from the Basidiomycetes

The phylogenetic relationships of the yeasts which form no spores cannot be determined with certainty, as with other imperfect fungi It has been widely assumed in the past that all of them are Ascomycetes which have permanently lost the power to form ascospores Among the asporogenous yeasts, however, the group forming rose, red or orange pigments show characteristics which separate them from the others These have been especially noted by Lodder (1934) who proposed to place them in a separate family, the *Rhodotorulaceae* We shall consider the characteristics of this family in a later section Nadson and Philippov (1928) observed the origin of yeasts resembling *Sporobolomyces* species from asporogenous mucoid rose yeasts indistinguishable from *Rhodotorula* species, and Derx (1930) observed the same sort of transformation to occur Lodder (1934) records that cultures of *Sporobolomyces* maintained in the type culture collection at Delft have irreversibly lost the power to form basidiospores and become indistinguishable from the ordinary rose asporogenous yeasts These facts indicate strongly that the common red yeasts are to be considered as imperfect forms of the basidiospore-forming yeasts of the genus *Sporobolomyces*

Unicellular growth forms appearing either as budding yeast-like cells or as oidia multiplying by fission occur so frequently in the growth of many different species of fungi of all classes that it seems improbable that those which are permanently or dominantly unicellular, and which we call yeasts, can be phylogenetically homogeneous Many species of the *Mucorales* give rise to budding yeast-like cells when grown submerged in liquid media, and it is noteworthy that such species also produce an alcoholic fermentation Nevertheless there are no true yeasts known to show relationships to the Phycomycetes Many of the fungi pathogenic to man, as *Blastomyces, Coccidioides, Histoplasma, Sporotrichum,* grow as yeast-like unicellular fungi in the tissues, but grow as mycelial fungi in artificial cultures, unless (in some cases) they are cultured in the presence of blood or serum under reduced oxygen tension

The known yeasts appear to fall rather sharply into two narrow and apparently monophyletic groups. Those which produce carotinoid pigments fall either into the basidiomycetous family of *Sporobolomycetaceae* or the imperfect family of *Rhodotorulaceae*. Those without carotinoid pigments fall in the ascomycetous family of *Endomycetaceae* or the imperfect family of *Torulopsidaceae*. The systematic relationships of the yeasts may be indicated by the following diagram:

ASCOMYCETES Family *Endomycetaceae*	BASIDIOMYCETES Family *Sporobolomycetaceae*
↓	↓
Family *Torulopsidaceae*	Family *Rhodotorulaceae*
F U N G I	I M P E R F E C T I

CLASSIFICATION OF YEASTS

The remainder of this review will deal with the classification and identification of yeasts, presenting first an outline of the systems of Stelling-Dekker (1931) for the ascospore-forming yeasts, and of Lodder (1934) for the asporogenous yeasts. This will be followed by discussions of selected groups of yeasts in which important taxonomic work has been done during the past decade. The review cannot be considered complete, since the technological literature has not been covered adequately. Undoubtedly, in the literature on dairy products, on silage, on foodstuffs, on the various fermentation industries, there may be found important taxonomic data which the reviewer has overlooked. Before considering the classification and identification of yeasts it is necessary to take some note of the various characteristics of yeasts by which they are classified and identified, and of the technical procedures which must be used to demonstrate these characteristics.

Technical Procedures for the Identification of Yeasts

The identification of yeasts involves a determination of both morphological and cultural characters. Of the morphological

characters it is necessary to determine the relative size and shape of the vegetative cells, whether they multiply by fission or by budding, if by budding, whether buds are formed at any place on the cell (multipolar) or only at the ends of elongated cells (bipolar), whether the buds are separated from the mother cell by abstriction or by the formation of a cross-wall followed by fission Further, it is necessary to determine whether the buds separate quickly after they are formed, giving rise to free cells or groups of only two or three, or whether they tend to remain attached forming clusters ("sprossverbande"), in the latter case the cells, if elongated, may give rise to a branched structure resembling closely true mycelium ("sprossmyzel," pseudomycelium), in some cases true mycelium with cross-walls is formed With yeasts forming pseudo- or true mycelium, clusters of yeast-like cells may arise by budding, usually at the nodes ("sprosskonidien," "blastospores," "appareil sporifère")

Since these vegetative characters do vary with the composition of the medium, it would be desirable if yeast workers would agree upon a standard reproducible medium upon which to grow yeasts for morphological descriptions The most widely used medium is beer wort, or beer wort agar This is not, however, a medium of standard composition, and not readily available to the laboratory which only occasionally works with yeasts Some workers have used hopped wort, others unhopped Workers in wineries have used must (grape juice), or must agar, Japanese workers have used "Koji" preparations Stelling-Dekker (1931) gives the following recipe for wort 1 kg of malt-meal (dried "Langmalz") is added to 2 6 liters of tap water and heated to 45°C for three hours with continuous stirring, it is then heated to 60°C for an additional hour, filtered, and the filtrate heated to 120°C for 15 minutes in the autoclave The filtrate is brought to a concentration of 15° Balling, again filtered, and finally sterilized The Digestive Ferments Company markets dehydrated wort agar which presumably is fairly constant in composition, but it is, in my opinion, too acid (pH 4 8) and when autoclaved, it is hydrolyzed partially and forms a soft, mushy medium I have compared the morphology of a limited number of yeasts

on wort agar and on agar containing 5 per cent pure glucose and 1 per cent Bacto peptone, and have found but slight differences The latter is a readily reproducible medium

The development of mycelium or pseudomycelium depends upon many factors, but especially upon the age of the culture and the nutrient value of the medium Talice (1930) has studied the conditions affecting the formation of pseudomycelium in the *Mycotoruloideae* He recommended especially the use of a dilute potato infusion (20 grams potato pulp to 1 liter of water) Three successive transfers in this medium should be studied before a yeast is to be considered free of mycelium formation Lodder (1934) used this method, incubating the cultures three weeks at 25° Rivalier and Seydel (1932) described a method for slide cultures which was also used by Lodder The medium consisted of 2 per cent dextrose, 1 per cent peptone, 2 per cent agar Martin, Jones, Yao and Lee (1937) used corn-meal agar in slide cultures Soriano (1938) used thin blocks of agar on the underside of coverslips sealed over the depression of concave-ground slides Some sort of slide culture is almost essential in clearly observing the morphology of the *Mycotoruloideae*

Benham (1931) found that corn-meal agar is an important medium in studying the morphology of the *Mycotoruloideae*, especially for the development of the diagnostic chlamydospores of *Candida albicans* This medium is made of 62 5 grams of corn meal heated in 1500 ml water at 60° for an hour, filtered, 19 grams agar added, the mixture steamed in the Arnold sterilizer 1¼ hrs , filtered through cotton, tubed and autoclaved The reaction is pH 6 to 7

In addition to the morphology of the vegetative cells, the presence or absence of spores, their mode of formation, and the number and shape or structure of the mature spores are important diagnostic characters Basidiospores of *Sporobolomycetaceae* are readily detected, appearing as a powdery coat on the surface of the colony, and forming the typical mirror colony on the lid of the culture dish From the mirror colony a pure preparation of spores may be obtained for microscopic examination The production of ascospores by *Endomycetaceae* requires the use of special

media or methods Although ascospores may appear abundantly in ordinary cultures on rich media, especially with strains recently isolated from nature, they cannot be considered as absent until repeated attempts have been made to force their development upon special media

The use of blocks of plaster of Paris moistened with water or with dilute peptone solution, which has been the standard method for inducing spore formation in yeasts since Hansen, has been replaced often in recent years by Gorodkowa's agar (glucose 0 25 per cent, meat extract 1 per cent, NaCl 0 5 per cent) Stelling-Dekker (1931) reviewed the literature on various methods for inducing spore formation, and concluded that no one procedure is adequate, a number of methods must be used in succession before a yeast may be called asporogenous In addition to the plaster block and Gorodkowa's agar, she used potato and carrot plugs, Kufferath's "gelose farhydre," exposure to ultraviolet light, raisins and raisin agar, and for *Debaryomyces* species, growth on sterilized sausage The medium of Kufferath (1928, 1930) is prepared from malt hydrolyzed with H_2SO_4, neutralized with $CaCO_3$, to which is added agar, and NaOH to give an alkaline reaction, a series of such media of varying degrees of alkalinity are prepared, and the unknown yeast is inoculated on all of them Mrak and McClung (1940) noted that in some instances spores were formed on cucumber wedges, when they failed to develop on other media The reviewer has used with much success McKelvey's (1926) agar (dilute carrot infusion with $CaSO_4$) which has repeatedly revealed spores that failed to develop on Gorodkowa's agar or plaster blocks Niehaus (1932) used soil extract gelatin (1000 to 1500 grams garden soil extracted with 1000 ml water, filtered, 15 per cent gelatin)

Ochmann (1929) made an extensive study of the influence of the nitrogen sources in the medium upon spore formation in six yeasts (*Saccharomyces cerevisiae* (Johannisberg strain), *S turbidans*, *S pastorianus*, *S validus*, *Schizosaccharomyces octosporus*, and *Schizosaccharomyces pombé*) Spore formation was induced by transferring the yeasts from the media studied to blocks of soaked and sterilized beechwood The basal medium was 10 per cent dextrose, 0 5 per cent KH_2PO_4 and 0 25 per cent

$MgSO_4$ To this was added 26 different nitrogenous compounds Wort was used as a control medium It was found that with each species spore formation was stimulated by certain N compounds which would not necessarily permit of spore formation with other species Thus nitrates favored spore formation by *S pastorianus* but not by the others, and so on This paper is cited to indicate the difficulties inherent in a classification of yeasts based upon spore formation Probably with many of the asporogenous yeasts we have not yet found the environmental conditions necessary for sporulation

Since spore-formation is often delayed, cultures should be examined repeatedly over a period of several weeks before they are to be labelled as asporogenous Windisch (1938) reported spore formation in *Torulopsis pulcherrima* only after the agar cultures had dried considerably, and Todd and Herrmann (1936) had the same experience with *Debaryomyces neoformans* Fuchs (1935) found that with old laboratory strains which had quit forming spores this character could be revived by growing them on wort agar, transferring the young growth to plaster blocks, then back to wort agar, and so on Usually after three such treatments, spore formation will begin again

By using aerated solutions, Stantial (1935) showed that washed cells of *S cerevisiae* could be made to sporulate in the presence of certain sugars, mannose or maltose gave up to 85 percent ascus formation Acetate also favored the process, especially in the presence of mannose or dextrose The yield was found dependent upon the ratio of cell to solute concentration, also, prior cultivation of the yeast in a bios-containing medium was a prerequisite for sporulation in the above procedure

The presence or absence of spores is determined by microscopic examination of wet preparations and of stained slides Recognition of spores in the living cells requires experience Often fat-globules or water-vacuoles have been mistaken for spores Spores may be differentially stained by one or another modification of Moeller's spore stain, widely used to stain bacterial spores The following method has given the best results in my hands 5 per cent chromic acid solution 5 minutes, Ziehl's carbol fuchsin steaming 5 minutes, decolorization for 2 minutes in 1 per cent

aqueous H_2SO_4, counterstain with Loeffler's methylene blue 1 minute Spores are red, asci and vegetative cells blue Ochmann (1929) stained the spores of *Schizosaccharomyces* species with methylene blue followed by Bismarck brown (spores blue, cells brown) and by Gram's method (spores blue-black, cells red) Kufferath (1928) insisted that differential staining should be the only criterion of spores, but many others have noted that in some yeasts the spores may fail to show the differential staining This has been my experience with *Nadsonia fulvescens* and with *Debaryomyces* species

In the genus *Debaryomyces* an important diagnostic character is the warty character of the wall of the mature spore This is not always present, and may be difficult to observe, largely because the single spore completely fills the ascus, and so the outer surface of the spore wall is closely applied to the inner surface of the ascus wall Mrak and Bonar (1938b) have observed that if the cultures inoculated in Gorodkowa agar with yeasts of this genus are incubated at 16°, or lower, the asci are larger in relation to the size of the ascospores, and the warty walls may be readily observed

The mode of growth of yeasts in liquid media is of greater diagnostic importance than in bacteria The formation of a dry, matte, pellicle from the beginning of growth is characteristic of the genera *Pichia*, *Hansenula*, some species of *Debaryomyces* and *Mycoderma* A mucoid scum may develop late with other genera This pellicle may be observed on the liquid media used for sugar fermentations Most recent descriptions of yeasts have been accompanied by illustrations of their giant colonies, usually grown upon wort gelatin It has been repeatedly demonstrated that the characteristics of these colonies are very easily changed by slight variations in the composition of the medium, especially the consistency of the substrate Here a standardized medium is especially desirable Stelling-Dekker (1931) rejected the giant colony in favor of the wort-agar stroke culture, as giving essentially the same sort of information The growth may be mucoid, pasty or tenacious, smooth, matte, or wrinkled, white, creamy, buff, or definitely colored

Lodder (1934) separated the asporogenous yeasts with carotinoid pigments in a family, the *Rhodotorulaceae*, and used a method devised by Molisch for the determination of the carotinoid pigment A considerable amount of growth is brought into a test-tube containing 2 ml of a 20 per cent solution of KOH in 40 per cent alcohol, and allowed to stand in the dark for some days Some of the sediment is removed with a pipette and examined microscopically for red or organge-red crystals In all cases where definitely red, orange or yellow yeasts were investigated, such crystals were found. Mrak and McClung (1940) experienced considerable difficulty with this method, and found that storage of the tubes for six weeks was necessary to give consistent results However, it is hardly necessary to resort to microchemical methods Lodder found no crystals from old cultures of nonchromogenic yeasts giving the yellowish-brown color that often appears with age The carotinoid pigments appear early in growth and are usually distinctive enough to be recognized by simple inspection of the culture tubes The "iron-red" pigment formed by *Torulopsis pulcherrima* and some other species is to be distinguished from the carotinoid pigments It is definitely maroon rather than pink, coral, or rose, as in the *Rhodotorulaceae*, it usually diffuses into the medium, and often fails to color the cells, which remain white It will not develop on synthetic media free from iron

In a number of genera of yeasts the species are identified by sugar fermentations Stelling-Dekker (1931) has discussed extensively methods for determining sugar fermentations She used two methods growth in Einhorn fermentation tubes containing 2 per cent of the sugar to be tested in yeast infusion (200 grams compressed yeast extracted in 1 liter of water in the autoclave), and quantitative determinations by used of the van Iterson-Kluyver apparatus, the latter method especially in studying the fermentation of raffinose

The fermentation of raffinose occupies a special place in Stelling-Dekker's system Some yeasts split this trisaccharide to levulose and the disaccharide melibiose, fermenting the levulose but not attacking the melibiose, such yeasts are described as fermenting

"$\frac{1}{3}$ of raffinose" Other yeasts completely hydrolyze raffinose to its component monosaccharides, and are said to ferment raffinose completely This procedure is therefore a method of separating yeasts upon the basis of their ability to ferment melibiose Stelling-Dekker's method requires the use of quantitative apparatus, an inconvenience in routine work I have been unable to determine why she does not study the fermentation of melibiose directly, its cost is considerably higher than that of raffinose, but not prohibitive Zimmermann (1938), to use Stelling-Dekker's keys, determined the character of the fermentation of raffinose by titrating the remaining sugar after 14 days

Generally, 2 to 3 per cent solutions of sugars and large volumes are used rather than the 1 per cent solution in small fermentation tubes commonly used to study fermentations by bacteria I have been unable to find any discussion of this, but there is a real reason for it I have repeatedly observed yeasts to give a vigorous fermentation in tubes containing 10 to 12 ml of liquid, when they gave no or slight fermentation in small tubes containing only 2 to 3 ml I suspect this to be due to more complete diffusion of oxygen in the smaller volume of liquid, favoring oxidative rather than fermentative respiration Martin, Jones, Yao and Lee (1937) recommend a vaseline seal on the sugar fermentation tubes to maintain anaerobiosis Langeron and Guerra (1938) used paraffin with paraffin oil

The reviewer uses, for fermentation tests, tubes of large diameter (25 x 150 mm) with inverted Wassermann tubes (10 x 75 mm) for gas-traps, containing about 20 ml of medium With pellicle-forming yeasts it is well to shake the pellicle free after a day or two, so that some cells will sink to the botton of the tube One should shake the tubes vigorously before reading the results, as sometimes the solution may be supersaturated with gas, yet show none in the gas-trap On shaking, effervescence is seen Yeasts often form acid without gas from sugars, these fermentations have so far found no place in the systematics of yeasts, save that numerous authors have noted that *Candida albicans* regularly produces acid from sucrose, though it produces alcohol and gas from dextrose and levulose

In 1914 Kluyver published certain generalizations concerning sugar fermentations by yeasts which were restated by Stelling-Dekker, and are referred to by Langeron and Guerra (1938) as the "Kluyver-Dekker laws", 1 A yeast which cannot ferment glucose cannot ferment any other sugar 2 A yeast which can ferment glucose can also ferment fructose and mannose 3 A yeast cannot ferment both lactose and maltose

In addition to fermentations, it is necessary with some groups of yeasts to determine what substances they may utilize as sources of nitrogen or carbon One prepares a basal medium containing all ingredients necessary for growth, including in one case a universally available source of carbon (all yeasts can utilize dextrose), in the other case a universally utilizable source of nitrogen (ammonium sulphate could be utilized in the presence of dextrose by all of the yeasts studied by Lodder) Other salts as potassium phosphate and magnesium sulphate are necessary If liquid media are used, the trace elements and growth-accessory substances ("bios" complex) must be added, which are easily provided by adding a trace of yeast extract to the medium

Lodder (1934) made use of Beijerinck's "auxanographic" method for testing the utilization of sugars by yeasts A basal medium is made, composed of 0 05 per cent $(NH_4)_2SO_4$, 0 1 per cent KH_2PO_4, 0 05 per cent $MgSO_4$, and 2 per cent of washed agar This is heavily seeded while molten with the yeast to be tested, and poured into a Petri dish The heavy seeding obviates the necessity of providing "bios" Small quantities of the sugars to be tested are deposited upon the surface of the agar in dry form, including dextrose on each plate as a control Where the sugar diffuses into the agar, growth will occur if this sugar can be utilized by the yeast being studied For testing the utilization of N-sources by the auxanographic method, Lodder's basal medium is 2 per cent dextrose, 0 1 per cent KH_2PO_4, 0 05 per cent $MgSO_4$ and 2 per cent washed agar Peptone, ammonium sulphate, asparagin, urea and potassium nitrate were tested as sources of nitrogen Zimmermann (1938) found the auxanographic method to give doubtful results, and preferred to use liquid synthetic media, as did also Mrak and McClung (1940)

Stelling-Dekker (1931) stressed especially the utilization of nitrates and of alcohol in identifying the spore-forming yeasts The utilization of alcohol was tested in a liquid medium containing 3 per cent ethyl alcohol, 0 1 per cent $(NH_4)_2SO$, 0 1 per cent KH_2PO_4 and 0 05 per cent $MgSO_4$ The utilization of nitrates was tested by inoculating simultaneously two tubes of agar, one composed of 2 per cent dextrose, 0 1 per cent KNO_3, 0 1 per cent KH_2PO_4, 0 05 per cent $MgSO_4$, and 2 per cent washed agar, the other tube of the same composition with nitrate omitted A slight growth almost always occurs in the control tube, but a definitely heavier growth in the tube containing nitrate indicates that this substance can be utilized

Classification of the Sporobolomycetaceae

The basidiospore-forming yeasts were united by Derx (1930) into a family of "Sporobolomycetes" described as follows "Microscopic fungi which mostly propagate by budding like *Saccharomycetes*, from part of the vegetative cells there are produced single or rarely branched, often bifurcated sterigmata projecting into the air, on which are formed light, hyaline, distinctly apiculate spores, which when mature are projected in a manner similar to the multiple spores of true Basidiomycetes The spores, which in form are similar to multiple basidiospores, may form secondary spores like the multiple basidiospores of *Protobasidiomycetes*" Within this family Derx included two genera, *Sporobolomyces* and *Bullera* (amended to *Bulleria* by Ciferri and Verona, 1938)

The genus *Sporobolomyces* is defined by Derx as follows "Vegetative growth rose, red or salmon Spores more or less compressed laterally, kidney-shaped, pear-shaped, asymmetric" The genus *Bulleria* is defined as "Vegetative growth white, whitish, cream, straw or yellow, without any trace of red Spores round, ovoid, globular, symmetrical" Derx recognized seven species of *Sporobolomyces* in a collection of 40-odd strains These were distinguished by the color of the vegetative growth, the shape of the spores, the texture of the colonies, and the odor of the cultures Ciferri and Verona (1938) proposed to subdivide

the genus into two groups, the subgenus *Blastoderma* with pseudomycelium, and the subgenus *Eusporobolomyces* without pseudomycelium They added several additional species There are but two species of the genus *Bullera* described, differing in the size of the vegetative cells, and the size and shape of the spores.

Classification of the Ascosporogenous Yeasts

Stelling-Dekker includes all of the yeasts which form ascospores, together with the related yeast-like (mycelial) forms in a single family, the *Endomycetaceae*, which is subdivided into four subfamilies, the *Eremascoideae*, the *Endomycoideae*, the *Saccharomycoideae* and the *Nematosporeae* The common spore-forming yeasts are mostly included in the third subfamily, which is further divided into three tribes, the *Endomycopseae*, the *Saccharomyceteae* and the *Nadsonieae* The distinguishing characters of these subfamilies and tribes are as follows

Family Endomycetaceae

Growth-forms mycelium, pseudomycelium, oidia or yeast cells ("conidia"), together or singly Vegetative multiplication by transverse fission or by budding Naked asci result from isogamous or heterogamous conjugation or parthenogenetic Ascospores spherical, hemispherical, angular, sickle- or spindle-shaped, smooth, warty, or with an encircling rim Both oxidative and fermentative species

Subfamily A *Eremascoideae*

Growth-form only mycelium Vegetative multiplication by transverse fission Spores hat-shaped, result from isogamous conjugation Dissimilation exclusively oxidative But one genus *Eremascus*

Subfamily B *Endomycoideae*

Growth-form either mycelium with oidia or only oidia Vegetative multiplication by transverse fission Spores round, oval or hat-shaped, result from isogamous or heterogamous conjugation Dissimilation oxidative or fermentative There are two genera, *Endomyces* with both mycelium and oidia, and both oxidative and fermentative respiration, and *Schizosaccharomyces* with no mycelium, only oidia multiplying by transverse fission, and with dominantly fermentative respiration

Subfamily C *Saccharomycoideae* Growth-form either mycelium with yeast cells ("conidia"), occasionally also oidia, or only budding yeast cells and then often pseudomycelium Vegetative multiplication by transverse fission, by multipolar budding or by bipolar budding, the latter upon a broad base Spores spherical, hemispherical, angular or sickle-shaped, or with an encircling ridge, formed by isogamous or heterogamous copulation or parthenogenetic All transitions between oxidative and fermentative dissimilation There are three tribes in this subfamily

Tribe A *Endomycopseae*

Growth-form mycelium with buds ("conidia"), at times oidia Vegetative multiplication by transverse fission and by multipolar budding Spores parthenogenetic or following isogamous copulation But one genus, *Endomycopsis* Dissimilation is dominantly oxidative, at times also fermentative

Tribe B *Saccharomyceteae*

No mycelium, only budding yeast cells or pseudomycelium Vegetative multiplication by multipolar budding Spores produced by isogamous or heterogamous conjugation, or parthenogenetic This tribe includes the largest number of species of spore-forming yeasts, contained in the genera *Saccharomyces, Torulaspora, Pichia, Hansenula, Debaryomyces*, and *Schwanniomyces*

Tribe C *Nadsonieae*

No mycelium, only budding yeast cells, at times pseudomycelium Vegetative multiplication by bipolar budding, more or less upon a broad base Spores parthenogenetic or following heterogamous conjugation There are three genera *Saccharomycodes* with round spores that conjugate during germination, *Hansienaspora* with parthenogenetic spores, and *Nadsonia*, in which following heterogamous conjugation between a bud and the mother cell, a second bud develops into an ascus

Subfamily D *Nematosporoideae* Growth-form mycelium and budding yeast cells Vegetative multiplication by multipolar budding Spores needle- or spindle-shaped, with or without flagella, parthenogenetic or formed after isogamous conjugation Both oxidative and fermentative There are three genera *Monosporella* with one needle-shaped spore, *Nematospora* with 2 to 8 flagellated spindle-shaped spores produced parthenogenetically, and *Coccidiascus* with 8 spindle-shaped non-flagellate spores produced by isogamous conjugation

We cannot, of course, take space to indicate the characteristics and activities of all of the genera of ascospore-forming yeasts, but a translation of Stelling-Dekker's key will serve to indicate the characters upon which their identification is largely based

Key to the genera of Endomycetaceae

1	a	Spores fusiform	[2]
	b	Spores not fusiform	[4]
2	a	Spores always single	*Monosporella*
	b	At least 4 spores per ascus	[3]
3	a	Spores with one non-motile flagellum	*Nematospora*
	b	Spores without flagella	*Coccidiascus*
4	a	Vegetative multiplication by transverse fission	[5]
	b	Vegetative multiplication by transverse fission, by multipolar budding, or both	[6]
	c	Vegetative multiplication by bipolar budding with more or less broad bases to the buds	[11]
5	a	True mycelium and oidia	*Endomyces*
	b	Only oidia, no mycelium	*Schizosaccharomyces*
6	a	True mycelium with crosswalls and "bud-conidia," at times oidia	*Endomycopsis*
	b	No mycelium, at times pseudomycelium, "bud conidia," no oidia	[7]
7	a	In wort a dry matte pellicle from the beginning	[8]
	b	In wort no pellicle, or a soft slimy pellicle after some time	[10]
8	a	Nitrates utilized (Lodder (1932) described a new yeast having the characters of *Hansenula*, but producing hat-shaped spores after isogamous conjugation, she therefore created a new subgenus, *Zygohansenula*, the type species being *Z californica*)	*Hansenula*
	b	Nitrates not utilized	[9]

9 a Spores round, angular or hat-shaped, smooth, cells in young cultures long-oval to filamentous *Pichia*
aa Spore-formation parthenogenetic
Subgenus *Pichia sensu strictu*
bb Spore-formation sexual
Subgenus *Zygopichia*
b Spores round with a warty wall, cells in young cultures round to short-oval *Debaryomyces*[1]
10 a Cells short-oval to elongated, spores round, kidney-shaped or hat-shaped, smooth, 1 to 4 per ascus *Saccharomyces*
aa Spore-formation parthenogenetic
Subgenus *Saccharomyces sensu strictu*
bb Spore-formation sexual
Subgenus *Zygosaccharomyces*
b Cells round, spores round and smooth, with an oil drop in the middle, copulation tubes formed before spore-formation *Torulaspora*
(Krumbholz (1933) questions the desirability of retaining the genus *Torulaspora*)
c Cells mostly round, but also oval, small Spores round with a warty wall, almost always 1, rarely 2 per ascus, formed sexually *Debaryomyces*[1]
d Cells oval, rather large, spores round, with a warty wall and a ridge around the middle, copulation tubes formed *Schwanniomyces*
11 a Cells small, bipolar budding, spores hat-shaped, smooth, 2 to 4 per ascus, parthenogenetic *Hanseniaspora*
b Cells large, bipolar budding on a broad base, spores round to oval, smooth, 1 to 4 per ascus Spores conjugate on germination *Saccharomycodes*

[1] This name was spelled *Debaromyces* in Tanner's translation of Guilliermond (1920) and this spelling has been used by American authors

c Cells large, bipolar budding on a broad base, spores round with a warty wall, one spore per ascus Conjugation before spore-formation, the ascus is formed by budding from the fertilized cell — *Nadsonia*

In order to indicate the characters upon which species are separated in Stelling-Dekker's system, the reviewer has also translated her key to the most important group, the subgenus *Saccharomyces* in the strict sense

Key to the species of Saccharomyces sensu strictu

1 a Fermentation of dextrose and galactose only — [2]
b Fermentation of dextrose, sucrose, and ⅓ raffinose only — [4]
c Fermentation of dextrose, sucrose and raffinose (completely) only — *S microellipsodes*
d Fermentation of dextrose, galactose, sucrose and ⅓ raffinose only — [5]
e Fermentation of dextrose, sucrose and maltose only — *S heterogenicus*
f Fermentation of dextrose, sucrose, maltose, and ⅓ raffinose only — [6]
g Fermentation of dextrose, galactose, sucrose (weakly) and maltose only — *S Chodati*
h Fermentation of dextrose, galactose, sucrose, maltose and ⅓ raffinose only — [7]
i Fermentation of dextrose, galactose, sucrose, maltose and raffinose (completely) only — [8]
j Fermentation of dextrose, galactose, sucrose, ⅓ raffinose, and lactose only — *S fragilis*

2 a Rather long clusters of cells in wort from the beginning — *S dairensis*
b Cells in wort singly or in pairs — [3]

3 a But one spore per ascus — *S unisporus*
b Several spores per ascus — *S globosus*

4	a	Cells elongated, in clusters	*S muciparus*
	b	Cells round or oval	*S Chevalieri* and varieties *Lindneri*, *torulosus*
5	a	Cells in young wort culture small, round to oval (3 5 to 5 5) μ x (4 to 7) μ	*S exiguus*
	b	Cells in young wort culture oval (3 5 to 5) μ x (5 to 10) μ	*S mangini* and variety *tetrasporus*
6	a.	Cells oval or egg-shaped	*S oviformis*
	b	Cells long-oval to elongated	*S Bayanus*
7	a	Cells in young wort culture round, oval, egg-shaped or pear-shaped, (3 to 7) μ x (5 to 14) μ, length to breadth ratio, 1 to 2	*S cerevisiae* and varieties *ellipsoideus*, *turbidans*, *Marchalianus*, *pulmonalis*, *festinans*
	b	Cells in young wort culture oval to sausage-shaped, (2 5 to 4) μ x (9 to 11) μ, length to breadth ratio, 3 to 4	*S intermedius*
	c	Cells in young wort agar short to long oval, (4 to 8) μ x (9 to 18) μ	*S Willianus*
	d	Cells in young wort culture oval, (3 to 5 5) μ x (5 to 9) μ, but elongated on wort agar (2 to 4 5) μ x (6 to 14) μ	*S odessa*
	e	Cells in young wort culture elliptical or elongated, (3 to 5) μ x (7 to 10) μ, on wort agar after a longer time very long, up to 30 μ, fermentation of maltose weak	*S tubiformis*
	f	Cells in young wort culture round to oval, (3 to 6) μ x (4 5 to 9) μ, in older cultures elongated, in old wort agar cultures forming irregularly shaped cell-complexes	*S paradoxus*

8	a	In young wort cultures long clusters of long-oval cells	*S pastorianus*
	b	Cells in young wort cultures single or in pairs or threes	[9]
9	a	Cells in young wort culture oval, (3 to 5) μ x (7 to 10) μ	*S carlsbergensis* and varieties *monacensis, valdensis, mandshuricus, polymorphus*
	b	Cells in young wort cultures filamentous, length to breadth ratio, 4 to 6	*S validus*
	c	Cells in young wort cultures long-oval (4 to 5 5) μ x (9 to 13) μ	*S Logos*
	d	Cells in young wort cultures oval to elongated, (3 to 6) μ x (7 to 15) μ, on wort agar oval to filamentous, (2 to 5) μ x (4 5 to 20) μ	*S uvarum*

Classification of the Asporogenous Yeasts

Lodder (1934), excluding from the asporogenous yeasts all of those yeast-like fungi which form true mycelium, includes those which form pseudomycelium She distinguishes these structures as follows "*True mycelium* is composed either of long, non-septate filamentous, sometimes branched cells, or if septate, often branched filaments, in which the separate elements arise by the formation of cross walls in the filaments By pseudomycelium, I understand septate, often branched filaments, in which the usually somewhat elongated cells have arisen, one from another, by budding"

In practice this is going to be an exceedingly difficult distinction to make One may readily recognize typical true mycelium and typical pseudomycelium, but one encounters many types of filamentous structures where the origin of the cells (by budding or by cross-walls) could be determined only by watching the filament grow Further, one frequently finds both true and pseudomycelium in a single pure culture, sometimes one and sometimes the other dominating, according to the age of the culture or the

composition of the medium We have noted already that the rough variants of yeasts may form true mycelium Stelling-Dekker makes no such distinction, frankly including with the yeasts fungi that form true mycelium but which multiply dominantly as yeast-like growth forms, and in a later publication, Diddens and Lodder (1939a) admit of some true mycelium in the *Mycotoruloideae*

Lodder subdivides the asporogenous yeasts into three families The family *Nectaromycetaceae* of Ciferri and Redaelli (1935) is retained to include the single genus *Nectaromyces*, the peculiar yeast found in the nectaries of flowers which sometimes forms conidia on the surface of the colony She excludes from this family the genera *Sporobolomyces* and *Bulleria* which Ciferri and Redaelli had included, on the ground that these yeasts form basidiospores, not conidia

The *Rhodotorulaceae* form a new family created to include all of those asporogenous yeasts which form carotinoid pigments As has been indicated, these may be imperfect forms of the *Sporobolomycetaceae*

The remaining asporogenous yeasts are contained in the family *Torulopsidaceae* These parallel to a considerable extent the ascospore-forming yeasts, and undoubtedly are for the most part imperfect forms of them A number of the genera of the asporogenous yeasts find a counterpart in the sporogenous group Thus the asporogenous lemon-shaped yeasts of the genus *Kloeckera* differ from the apiculate sporogenous yeasts of the genus *Hanseniaspora* only in the matter of spores, and the elongated pellicle-forming yeasts of the genus *Mycoderma* are obviously the asporogenous forms of the sporogenous genus *Pichia* From time to time, as with other imperfect fungi, spores are found in yeasts formerly believed to be asporogenous Recent observations of this sort are the discovery of spores in a strain of *Torulopsis pulcherrima* by Windisch (1938), and the observation by Niehaus (1932) and by Dvornik (1938) that all strains of *Kloeckera* form spores when freshly isolated Diddens and Lodder (1939b) record the discovery of spores in the following yeasts formerly reported as asporogenous, with the changes in nomenclature in-

dicated *Monilia pinoyisimilis* and *Monilia pseudotropicalis* = *Saccharomyces fragilis*, *Monilia macedoniensis* = *Saccharomyces macedoniensis*, *Monilia javanica* = *Hansenula anomala*, *Candida pelliculosa* = *Hansenula javanica* Todd and Herrmann (1936), confirmed by Giordano (1939), noted that many strains of pathogenic yeasts formerly referred to as *Torula histolytica*, *Torula hominis*, etc. actually form spores of such a character as to warrant referring them to the genus *Debaryomyces*

Balţatu (1939) claimed that he could induce sporulation in all of the strains of *Mycoderma* which he studied (6 from the Botanical Institute of Geisenheim, 7 from the Centraalbureau voor Schimmelcultures, and one isolated by himself) by growth in grape must, or on plaster blocks moistened with grape must, especially if the medium was acidified One to eight round spores were formed, usually two or four according to the species At times spore-formation was preceded by copulation between two vegetative cell, at times the spores fused on germination In the latter case fusion of four or more spores resulting in but one vegetative cell was observed! He would transfer the yeasts designated *Mycoderma* to the ascosporogenous group, and would include all of the pellicle-forming yeasts in one genus, *Mycoderma*, with three subgenera *Mycoderma sensu strictu* with round spores, *Pichia* with hemispherical spores, and *Willia* (= *Hansenula*) with hat-shaped spores A number of species are described, some with new names

The *Torulopsidaceae* are subdivided into two sub-families The *Torulopsidoideae*[2] include those yeasts with no (or only primitive) pseudomycelium, and no "appareil sporifère" (clusters of buds arising at the nodes of the pseudomycelium) The *Mycotoruloideae* form pseudomycelium and an "appareil sporifère". The latter subfamily is to be the subject matter of part II of her study It includes yeast-like fungi which have been the subject of an enormous, confused and contradictory literature These yeasts will be omitted from this review, hoping that soon the publication of Miss Lodder's second volume will provide a sound basis for the study of this group

[2] Miss Lodder in her work of 1934 used the name *Torulopsoideae*, but Diddens and Lodder (1939a) point out the correct spelling

The *Torulopsidoideae* comprise the majority of the "wild yeasts", common as contaminants in all sorts of bacteriological work

Key to the genera of Torulopsidoideae

1 a Cells mostly lemon-shaped, bipolar budding *Kloeckera*
  b Cells mostly triangular, budding at the three angles *Trigonopsis*
  c Cells mostly flask-shaped, budding often upon a broad base *Pityrosporum*
  d Cells otherwise, usually round, oval or cylindrical [2]
2 a No pellicle in wort cultures, or only a soft slimy pellicle after some time [3]
  b A matte, dry pellicle in wort cultures from the beginning [4]
3 a Formation of long slender tubular processes resembling the copulation tubes of *Zygosaccharomyces* on Gorodkowa agar *Asporomyces*
  b No tubular processes formed *Torulopsis*
4 a Cells often cylindrical, multiplication by budding, the buds not separated from the mother-cell by fission *Mycoderma*
  b Cells polymorphous, multiplication by budding, the buds often separating from the mother-cell by fission *Schizoblastosporion*

The largest number of species of the *Torulopsidoideae* are to be found in the genus *Torulopsis* There has been some argument regarding the proper scientific name of this genus The name *Torula* widely applied since the time of Pasteur and Hansen is invalid since it had been applied previously by Persoon in 1801 to an entirely different sort of fungus The name *Cryptococcus mollis* was given by Kützing in 1833 to an organism which he found on moist window panes and which he classified with the algae Later he included in this genus *Cryptococcus fermentum* and *Cryptococcus cerevisiae*, obviously yeasts This name was not, however, applied generally to yeasts until it was revived in 1901 by Vuillemin, who redefined the genus to include only

pathogenic yeasts, since which time it has been widely used by medical mycologists It is agreed, however, that pathogenicity is too indeterminate and variable a character for generic determination Benham (1935) reexamined some of the herbarium material of Kutzing deposited in the New York Botanical Garden, and found a variety of organisms, including budding cells which she considers to be yeasts She therefore accepted *Cryptococcus* as having priority as a generic name for asporogenous yeasts Kutzing had not described budding

Lodder (1938) examined some of Kutzing's original material deposited in the Rijksherbarium at Leyden and also found a mixture of organisms with some budding cells She pointed out, however, that these do not fit Kutzing's description, and rejected *Cryptococcus* as being both a *nomen dubium* and *nomen confusum*, and accepted the name *Torulopsis* introduced by Berlese in 1895 as a substitute for the inadmissible name *Torula*

The following key to the species of *Torulopsis* will indicate the characters by which the species are distinguished

Key to the species of Torulopsis

1	a	Fermenting sugars	[2]
	b	Not fermenting	[8]
2	a	Fermenting only dextrose	[3]
	b	Fermenting dextrose and sucrose	[4]
	c	Fermenting dextrose, galactose and sucrose	*T Holmii*
	d	Fermenting dextrose, sucrose and maltose	*T colliculosa*
	e	Fermenting dextrose, sucrose and lactose, cells long-oval	*T kefyr*
	f	Fermenting dextrose, galactose (very weakly), sucrose and lactose, cells round	*T sphaerica*
3	a	Agar stroke culture not slimy, formation of a reddish pigment in the presence of iron	*T pulcherrima* and var *variabilis*
	b	Agar stroke culture slimy, no red pigment	*T Molischiana*

4	a	Cells relatively large (3 5 to 5 5) μ x (5 to 12) μ	[5]
	b	Cells small (1 5 to 4) μ x (3 to 5) μ	[6]
5	a	Nitrates utilized	*T utilis*
	b	Nitrates not utilized	*T dattila*
6	a	Cells in wort cultures in clusters, only *peptone utilized*	[7]
	b	Cells in wort cultures single or in pairs, ammonium sulphate and asparagin utilized as well as peptone	*T Gropengiesseri*
7	a	Cells mostly oval to long-oval	*T bacillaris*
	b	Cells mostly round to oval	*T stellata*
8	a	Nitrates undoubtedly utilized	[9]
	b	Nitrates utilized but slightly or not at all	[11]
9	a	Wort agar stroke culture slimy	[10]
	b	Wort agar stroke culture not slimy	*T aeria*
10	a	Cells in wort and wort agar culture round to short-oval, stroke culture yellowish	*T alba* and var *japonica*
	b	Cells in wort round to short-oval, in wort agar oval, stroke culture yellowish tending toward red	*T rotundata*
11	a	Wort agar stroke culture slimy	[12]
	b	Wort agar stroke culture not slimy	[15]
12	a	Cells round to short-oval	*T neoformans*
	b	Cells oval or long-oval	[13]
13	a	Cells in young wort culture mostly oval, length to breadth ratio <2	[14]
	b	Cells in young wort culture mostly long-oval, length to breadth ratio >2	*T flavescens*
14	a	Lactose utilized, no growth with alcohol as source of carbon	*T Laurentii*
	b	Lactose not utilized, good growth with pellicle when alcohol is source of carbon	*T luteola*
15	a	Dextrose only utilized	*T uvae*
	b	Other sugars utilized	[16]
16	a	Cells round	*T candida*
	b	Cells oval	[17]

17 a Cells relatively large (3 5 to 5) μ x (5 to 11) μ *T lipofera*
b Cells relatively small (2 5 to 4) μ x (3 5 to 5) μ *T minor*

The Rhodotorulaceae

Lodder justified the creation of a new family to include the red yeasts on the ground that these form a homogeneous group, all producing carotinoid pigments which are lacking in other yeasts, none fermentative, and apparently related to the *Sporobolomycetaceae* rather than to the ascosporogenous yeasts, as has been indicated in an earlier section

Red or pink yeasts occur as air contaminants in bacteriological work probably more frequently than any other yeasts The reason for their abundance in the air is not known, for they certainly are not common in soil The observation of Derx that the Sporobolomycetes are abundant on leaves and straw may explain their origin Being rather inert biochemically, they are of little practical importance They are a cause of discoloration of sauerkraut, as reported by Pederson and Kelly (1938), who found that factors which tended to inhibit the normal bacterial sequence (high temperature, high salt content, high acidity, low nitrogen content) favored the growth of pink yeasts Schnegg and Weigand (1936) made the surprising observation that pink yeasts were present in numbers from 48 to 38,400 per ml in 11 samples of boric acid solution (3%) purchased in pharmacies

Lodder includes all of the *Rhodotorulaceae* in one genus, *Rhodotorula*, in which she recognizes 13 species and 10 varieties These are subdivided into two groups on the basis of their ability to utilize nitrates Within these groups the species are differentiated according to the shade of the pigment, the size and shape of the cells, and the sliminess of the growth on agar, characters which the reviewer considers to be highly variable *Rhodotorula glutinis* is the type, and most common species

Industrial Yeasts

Ever since Hansen applied the name *Saccharomyces cerevisiae* to a brewing yeast, and *Saccharomyces ellipsoideus* to a wine

is in their rates of fermentation and respiration In a study of 12 strains of top yeasts and 10 strains of bottom yeasts, they found the repiratory rate of the former to be on the average 77 per cent greater than the latter There was a constant ratio between the respiratory rate and the fermentation rate They consider these to be constant racial characteristics

As early as 1894 Bau had noted that various races of brewery yeasts differed with respect to their ability to hydrolyze and ferment melibiose, top yeasts failing to produce gas from this disaccharide while bottom yeasts do Stelling-Dekker (1931) confirmed this, and divided the industrial yeasts into two groups upon this basis As has been mentioned, instead of melibiose she uses raffinose and quantitative methods Top yeasts, of which *S cerevisiae* is the type, ferment raffinose only one-third, bottom yeasts, of which *S carlsbergensis* is the type, ferment this sugar completely

Hansen had noted as early as 1905 that top and bottom fermentation are variable characters He succeeded in developing top yeasts from bottom yeasts by growth at low temperatures, but could not accomplish the reverse change Winge and Laustsen (1939) obtained bottom yeasts from top yeasts by spore segregation They state that "it appears quite unjustified to use the ability to ferment the whole or one-third of raffinose as standards for distinguishing bottom and top yeasts respectively, as there is no regular connexion between these characters", and that "the categories of top yeast and bottom yeast have undoubtedly been interpreted too literally both in practice and in the literature "

Stockhausen has recently published a series of papers on what he calls "high-fermenting" and "low-fermenting" yeasts By these terms he apparently means something different from top and bottom yeasts, since he speaks (Stockhausen and Koch, 1936) of "hoch und niedrigvergärenden untergarige Bierhefen" The type of the high-fermenting yeasts is the Frohberg yeast (which Stelling-Dekker places in *S carlsbergensis*), the type of the low-fermenting yeasts is the Saaz yeast, which on the basis of raffinose fermentation is listed by Stelling-Dekker as *S cerevisiae* Stockhausen (1935) stated that the Saaz yeast is not a true cultivated

yeast, but a wild yeast, *S cratericus*, that gives beer a raw flavor and other undesirable characters

Stockhausen and Koch (1936) stated that the low-fermenting yeasts leave much more sediment when the brewing vat is drained than do high-fermenting yeasts This is apparently due to the flocculation and sedimentation of the former ("bruchhefen") and the fine distribution of the latter ("staubhefen") High-fermenting yeasts are carried over into the after-fermentation vats to a greater extent In laboratory tests no differences in the total yeast production could be noted Stockhausen (1935) stated that while one may speak of high-fermentation and low-fermentation as racial characteristics, these are strongly influenced by external factors Protein-rich wort leads to high fermentation Low-fermentation yeasts are rich in phosphorus and magnesium, high-fermenting strains contain more calcium In mixtures the high-fermenting yeasts predominate

Guilliermond (1920) stated that top yeasts ferment at higher temperatures than bottom yeasts This classification of the brewery yeasts may thus be parallel to the division of wine yeasts into "normal-fermenting" and "cold-fermenting" types "Cold" yeasts have been recently studied by Porchet (1936) and by Percher (1938) A slow but complete fermentation of must may be carried out by some of these yeasts even at 0°C The resulting wines are no different from those produced by normal wine yeasts Osterwalder (1934) made an extensive study of one such yeast, which he identified as *Saccharomyces intermedius*, var *turicensis* It fermented dextrose, levulose, galactose, sucrose, maltose and raffinose Zimmermann (1936) described a cold-fermenting yeast, and later (1938) two more, which differed from the "normal" wine yeasts in fermenting raffinose completely rather than one-third Mrak and McClung (1940) found but one of 195 strains of *Saccharomyces* from grapes, must or pomace which fermented raffinose completely

Several other procedures for differentiating industrial yeasts have been proposed Fink (1932) noted that yeasts of different sources vary in their cytochrome spectra Bakery yeasts, *i e*, compressed yeasts of the "respiratory" type, present the typical

4-band spectrum of reduced cytochrome Brewery yeasts, or yeasts of the fermentative type, present incomplete, usually 2-band spectra The test is easily and quickly carried out and can be used to detect the adulteration of commercial bakery yeast with brewery yeast Scum-forming yeasts show spectra of the "respiratory" type, wine and distillery yeasts those of the fermentative type The character of the spectrum depends upon the degree of aeration during the previous cultivation of the yeast Baker's yeast grown anaerobically was easily changed to one with an incomplete cytochrome spectrum, and wine and distillery yeasts yielded a 4-band spectrum when strongly aerated, but this was not observed with brewery yeast except after prolonged cultivation with a high degree of aeration (Fink and Berwold, 1933)

Stockhausen (1935) noted variations in the amount of gum produced by different types of industrial yeasts Beer yeasts produced 4 to 6 per cent, wine yeasts 6 to 8 per cent, and baking yeasts 8 to 12 per cent Drews (1937) noted that compressed yeast made from brewery yeasts did not keep as well as that made from selected baking strains This was due to the variations in pH optima for autolysis For a bottom brewing yeast this was 5 0, for a top brewing yeast, 4 7, for a top distillery yeast (grown under aeration as for compressed yeast manufacture) 4 25, and for a top baker's yeast grown on grain mash, 4 35

Schultz, Atkin and Frey (1940) propose to subdivide industrial yeasts according to their requirements for certain growth accessory substances They studied 44 strains, varieties or races of *S cerevisiae* and *S carlsbergensis*, and found that these fell into three groups The yeasts are grown upon a basal synthetic medium containing the bios ingredients (inositol, β-alanine, and Bios III), the same medium plus thiamin, and the same medium plus thiamin and vitamin B_6 Type A yeasts give a low crop on basal medium, increased by thiamin, and further increased by vitamin B_6 Type B yeasts give a high crop on the basal medium, which is depressed not more than 50 per cent by thiamin, and normal with both thiamin and vitamin B_6 Type C yeasts are depressed more than half by thiamin, but give a high crop with

both substances present They found all three types in both species, *S cerevisiae* (top) and *S carlsbergensis* (bottom)

The Genus Brettanomyces

Although properly belonging to the *Mycotoruloideae,* brief notice will be taken of the yeasts of the genus *Brettanomyces* because of their industrial importance They are concerned with the after-fermentation of English "stock" beers (porter, stout, pale ale) and are very different from the *Saccharomyces cerevisiae* responsible for the main fermentation Similar yeasts are used in the production of Belgian lambic beer Such yeasts were first studied in 1904 by Claussen who observed that they produce unusually high percentages of alcohol, and large amounts of both non-volatile and volatile acid They were further studied by Schiønning in 1907–1909, who recognized two types differing in morphology and cultural characters These two types were also recognized by Kufferath and van Laer in 1921 who proposed the generic name *Brettanomyces,* correcting "Brittanomyces" which had been used by Claussen Kufferath and van Laer described two species, *B bruxellensis* with moist, shiny colonies and *B lambicus,* with dull colonies like those of film yeasts Both were isolated from Belgian lambic beer

Very recently these yeasts have been intensively investigated by Custers (1940) He believed that such yeasts have been obtained only from English and Belgian beers, excepting one strain isolated by Krumbholz and Tauschanoff (1933) from a spontaneously fermenting French grape must, and named by them *Mycotorula intermedia* Custers considered this to be a species of *Brettanomyces* close to *B bruxellensis,* but differing from the others in a greater tendency to form pseudomycelium in all media, he suggests that it may be the "wild" form of these yeasts, the others having been modified by cultivation

Custers studied 17 strains, of which he isolated 7 from lambic beer, and 2 from English beer The remaining strains had been isolated by Schiønning, Kufferath, and by Krumbholz and Tauschanoff These strains were subjected to taxonomic analysis according to the procedures described by Stelling-Dekker and

Lodder The 17 strains fell into 4 species and two varieties Morphologically they are variable, but a characteristic feature is the "ogive" cell, elongated cylindrical cells pointed at the ends All tend to form some pseudomycelium in potato infusion Physiologically they differ from most yeasts in their ability to oxidize alcohol to acetic acid under aerobic conditions, producing enough acid to kill the cultures, so that stock cultures can be maintained readily only in media containing calcium carbonate They grow very slowly, taking nearly six months for the complete fermentation of beer wort, and producing about 10 per cent of alcohol

Custers proposes to include *Brettanomyces* as a genus of the tribe *Mycotoruloideae* which he defines as follows (in translation)

"Cells ovoid or globular, often elongated or "ogive" shaped, pointed at the ends Budding from all parts of the cell, forming irregular clusters Tendency to form a poorly developed pseudomycelium with only a primitive blastospore apparatus Ascospores are not formed In malt extract slow growth usually accompanied by slow, long drawn out fermentation, sediment, sometimes a pellicle In this medium and in malt agar, formation of a characteristic aroma Under aerobic conditions, strong production of acid from sugars Slow growth, relatively rapid death of cells in malt agar, but can be maintained longer in malt agar or yeast-extract glucose agar if calcium carbonate is added Can ferment a variety of sugars Potassium nitrate and nitrite (in dilute solutions), ammonium sulphate, urea and peptone can be utilized as sources of nitrogen"

The four species are *Brettanomyces anomalus* n s , *B Claussenii* n s , *B lambicus*, and *B bruxellensis*, there are two varieties of the last species, *non-membranaefaciens* and *lentus* These species and varieties are differentiated by sugar fermentations, by the character of the growth on wort agar, and by the presence or absence of pellicles

Custers made extensive chemical studies of the fermentations caused by species of *Brettanomyces* Under anaerobic conditions, only an alcoholic fermentation occurs, no appreciable amounts of acid are formed The amount of alcohol exceeds that of CO_2 in the classical formula for fermentation Under aerobic con-

ditions considerable acetic acid is formed This is thought to be due to an oxidation of the alcohol by the yeast In similar experiments with *Saccharomyces cerevisiae* and *S carlsbergensis* no acetic acid was formed At pH 6 4 the oxidation of the alcohol stops at acetic acid, at pH 4 35 and 3 77 the acetic acid is further oxidized to carbon dioxide and water It was further found that under aerobic conditions *B Claussenii* produces more alcoholic fermentation than under anaerobic conditions, *i e*, a negative Pasteur effect

Apiculate Yeasts

The apiculate yeasts are those with lemon-shaped cells (ellipsoidal cells with a small button-like projection at each pole) Such yeasts are common on grapes, and growing more rapidly than the true wine yeasts, are abundant at the beginning of fermentation They may contribute unfavorably to the flavor of the wine They have been studied considerably with rather confusing results as regards classification There has been dispute concerning spore formation, and we consequently find them listed with both the sporogenous and the asporogenous yeasts

Stelling-Dekker (1931) recognizes *Hanseniaspora* as the valid generic name for the sporogenous apiculate yeasts She defines the genus as follows "Cells lemon-shaped or long-oval, vegetative multiplication by bipolar budding No pellicle on wort Spores at first spherical, later hat-shaped, which is not always distinct Ability to form spores easily lost on prolonged cultivation Fermentation of dextrose, levulose and mannose only, at times weak Nitrate assimilation negative Almost no growth with ethyl alcohol as a source of carbon" She includes two species, *H valbyensis*, strongly fermenting and with never more than 2 spores per ascus, and *H Guilliermondi* with 4 spores per ascus

Lodder (1934) accepts *Kloeckera* as the valid generic name for the asporogenous apiculate yeasts, which she defines as "Cells lemon-shaped, short-oval, long-oval, or sausage-shaped Vegetative multiplication by bipolar budding Strong fermentation of dextrose (levulose and mannose) or of dextrose and sucrose Of the N-compounds tried, only peptone was utilized With

ethyl alcohol as the growth substrate, almost no growth" She describes ten species and one variety These are subdivided first on the basis of sucrose fermentation, further on the size and shape of the cells and the appearance of the agar-stroke culture

Niehaus (1932) published an extensive study of 81 strains of apiculate yeasts from widely distributed vineyards He noted, as had other previously, that the apiculate form is characteristic of young cultures, and that in older cultures ellipsoid or cylindrical cells are found When hanging-drop cultures were studied it was observed, however that newly-formed cells were ellipsoid, and only became apiculate when about to bud He explains the relative absence of apiculate cells in old cultures as due to the inhibition of budding by the accumulation of alcohol, to which the yeast is very sensitive, excepting one strain, only from ½ to about 3 per cent of alcohol being produced

Each of the 79 freshly isolated strains readily produced spores on plaster blocks One other formed spores on Kufferath's medium The spores were almost always single, only occasionally were two spores found Spore formation was parthenogenetic Nuclei were demonstrated in the spores, and they were observed to germinate, sending out a short promycelium from which vegetative cells were budded Since the form of the spores (no hat-shaped ones observed) and their germination appeared to be different from that described by Stelling-Dekker for *Hanseniaspora* he created a new genus, *Kloeckeraspora,* to include the yeasts which he studied To the reviewer this seems to be hair-splitting with a vengeance Castelli (1935) failed to confirm the observations of Niehaus He observed globular bodies corresponding to the single spores described by Niehaus, but his microscopic studies indicated that they were fat globules, and germination studies were negative

Dvornik (1938) reported (from the same laboratory as Niehaus) new extensive studies and fully corroborated the findings of Niehaus Each of 50 freshly isolated strains produced spores Only one spore per cell was formed parthenogenetically The identity of the spores was established by spore-staining, by the staining of nuclei within the spores, and by germination The promycelium described by Niehaus could not be confirmed

Saccheti (1939) also confirmed the regular formation of spores by 120 strains of apiculate yeasts freshly isolated from various fruits The ability to form spores was quickly lost on continued cultivation The spores could not be differentially stained

My curiosity piqued by reading these papers during the preparation of this review, I have recently obtained three samples of table grapes (Concord, white seedless, and Tokay) and allowed them to ferment after crushing At the beginning of fermentation plates were poured, and the majority of the colonies in all three samples proved to be apiculate yeasts Four or five were subcultured from each sample, and all of the 14 subcultures readily formed spores varying from 1 to 4 in number on Gorodkowa's agar They were all identified as *Hanseniaspora Guilliermondi* From 241 yeasts isolated from grapes, must or pomace, Mrak and McClung (1940) obtained 11 diagnosed as *Hanseniaspora,* 1 as *Kloeckeraspora,* and 16 as *Kloeckera* They observed that apiculate yeasts were isolated more frequently from grapes than from must or wine, and Zimmermann (1938) obtained only one apiculate yeast in 45 strains isolated from wines which had become turbid in storage The abundance of apiculate yeasts in must at the beginning of fermentation and their relative absence in the finished wine may be attributed to their sensitivity to alcohol, Niehaus (1932) found that most of them were inhibited by a concentration of about 3 7 per cent of alcohol in must

The existing confusion regarding the classification of the apiculate yeasts indicates clearly a difficulty frequently encountered when yeasts must be placed in different genera on the basis of spore-formation The imperfect yeasts are obviously an artificial (though necessary) category To the reviewer the balance of evidence at the present time indicates that all apiculate yeasts are potentially sporogenous, but that the ability to form spores is rapidly lost in artificial cultivation

Film-Forming Yeasts

Pellicle-forming yeasts fall in the genera *Hansenula, Pichia, Zygopichia* and *Debaryomyces* of the sporogenous yeasts, and in the genus *Mycoderma* of the asporogenous yeasts Growing as a

scum on the surface of liquid media, they tend to be oxidative rather than fermentative in the dissimilation of sugars If fermentative, they produce but little alcohol, tending to produce considerable amounts of esters Many of them utilize alcohol readily, and may be a cause of spoilage of fermented beverages, or may contribute to the development of special flavors, as in sherry Such yeasts also appear frequently in pickling solutions of various sorts, especially brines

The film of yeasts concerned in the development of flavor in sherry (and in certain Arbois wines of France) is called the "flor" Hohl and Cruess (1939) isolated 15 strains of film-forming yeasts from the mixed flora of samples of "flor" obtained from Jerez de la Frontera and from the Arbois district Three strains fell in the genus *Pichia*, very close to *P membranaefaciens* Seven strains fell in the genus *Saccharomyces*, very close to *S cerevisiae* var *ellipsoideus* These strains differ from the typical *Saccharomyces* species in forming a more or less heavy film, after fermentation is complete One strain proved to be *Hansenula saturna* Four strains were species of *Torulopsis* (closest to *T dattila*) They were actively fermentative, and could perhaps be strains of *S cerevisiae* which failed to form spores This paper presents an extensive bibliography on "flor" yeasts and discusses some of the physiological properties of the strains isolated Unlike the apiculate yeasts, they show a high alcohol tolerance, and tend to reduce the alcohol content of the wine

Scrivani (1939) also investigated the taxonomy of the "flor" yeasts He isolated 119 strains from 54 samples of Italian wines The following species were obtained *Zygopichia chantigrana*, *Pichia Derossii*, *Pichia membranaefaciens*, and its variety *acidificans*, *Mycoderma cerevisiae*, *Mycoderma Lafarii*, *Mycoderma vini*, *Mycoderma acidificans* var *degradans*, and 4 undetermined species of *Mycoderma*

Castelli (1933) found species of *Hansenula* to be the dominant yeasts in a sour-dough ferment used in household baking in Italy, and described two new species of the genus

Mrak and Bonar (1939) studied film-forming yeasts from pickle brines Yeasts were isolated from 29 food brines (dill

pickle, cucumber salt stock, Zucca melon, green olive, Sicilian olives, cauliflower and ham) The yeasts isolated were identified as *Pichia membranaefaciens*, *Mycoderma decolorans*, *Debaryomyces Guilliermondi* var *nova zeelandicus*, *Debaryomyces membranaefaciens*, and *D membranaefaciens* var *hollandicus* The salt tolerance of these yeasts was determined, and compared with that of related yeasts from type culture collections The *Debaryomyces* species were found to be more widely distributed in brines, and to show a higher salt tolerance than the other yeasts studied Though *Debaryomyces* species could grow in pickle brines containing up to 24 per cent salt, the *Mycoderma* and *Pichia* species were limited to brines containing 15 per cent or less The paper includes a bibliography on brine yeasts and their salt tolerances

Otani (1939) found that film-forming yeasts were the dominant species in "Nukamiso" pickles, a naturally fermented food prepared from raw vegetables in Japan Such yeasts were more frequent in the less acid samples, no yeasts were obtained from a highly acid sample Of the film-forming yeasts, *Hansenula anomala* and two unnamed species of *Mycoderma* are described In addition certain yeasts that did not form scums were isolated, among them *Zygosaccharomyces nukamiso*, n s , and *Torulopsis nukamiso*, n s

Mrak and Bonar (1938a) studied yeasts forming a slime on sausages Those isolated best fitted the description of *Debaryomyces Guilliermondi* var *nova zeelandicus* Difficulties were encountered in distinguishing between species of *D Guilliermondi* and *D membranaefaciens*

Osmophilic Yeasts

The term "osmophilic" was applied by Richter in 1912 to microorganisms which can multiply in solutions of high osmotic pressure The brine yeasts just mentioned could be well designated by this term In recent years there have been described another group of yeasts having a tolerance for high osmotic pressures, those growing in concentrated musts, honey or syrups

Krumbholz was concerned with yeasts which grow in concentrated musts In the Rhine wine region there occurs at times

a warm, dry, sunny autumn, when the grapes attain a characteristic over-ripeness designated as "edelreif" Such grapes may become infested with *Botrytis cinerea*, in which case they are designated as "edelfaul" From "edelreif" or "edelfaul" grapes there is obtained a must of unusually high sugar content, 30 to 40 per cent, sometimes as high as 60 per cent From such concentrated must there is produced an exceptional type of wine, the "rheinischen Ausleseweine" The fermentation is slower and less complete than that of ordinary wines Kroemer and Krumbholz (1931) presented a preliminary report upon yeasts isolated from such wines, and showed that the fermentation is brought about by species different from the *ellipsoideus* variety of *S cerevisiae* commonly concerned in wine production Yeasts were collected from naturally fermenting "Trockenbeerenauslesen," and grapes from various sources were placed in sterile concentrated must from which yeasts were isolated after fermentation had begun It was found that osmophilic yeasts were widely distributed upon "edelreif" and "edelfaul" grapes From 39 samples species of *Zygosaccharomyces* were isolated, from 14 samples small-celled budding yeasts, from 3 samples both of the preceding groups, from 2 samples apiculate yeasts, from 2 samples species of *Hansenula*, and from 2 samples species of *Rhodotorula* It was concluded that the yeasts active in the fermentation of the concentrated musts fall in the first two groups

The species of *Zygosaccharomyces* were divided into two specific groups one, related to *Z priorianus*, contained three new species, *Z polymorphus*, *Z variabilis*, and *Z amoeboideus*[3], the other, related to *Z Nadsonii*, contained one new species, *Z globiformis*. Species in the first group were found to be especially resistant to high osmotic pressures, growth continuing up to a concentration of 90 grams of sugar in 100 ml They grew and fermented best at temperatures above 20°, in sugar concentrations of 40 to 50 per cent, gave a slow fermentation with but little alcohol and no volatile acid *Z globiformis* was less tolerant of the higher osmotic pressures, equally weak in fermentation, and produced

[3] Lodder (1932) considers *Z amoeboideus* to be very close to *Z cavarae* var *beauverie* and suggests renaming it *Z cavarae* var *amoeboideus*

some volatile acid The small-celled budding yeasts were designated as species of *Saccharomyces* though no spores were found They fell into three groups designated as *S stellatus*, *S bacillaris*, and *S granulatus* (all new species) *S stellatus* grew in sugar concentrations up to 90 per cent with the optimum between 30 and 40 per cent, and produced up to 10 per cent alcohol The other two species gave weaker fermentations Extensive studies on the activities of these various yeasts in natural wine fermentations are presented

Krumbholz (1931a) described the morphology and cultural characters of the four new species of *Zygosaccharomyces* Several varieties or races of *Z polymorphus* were recognized Diagnostic characters of this species are the form of the giant colonies and the occurrence of long copulation tubes at the time of spore formation The isogamous (occasionally heterogamous) conjugation and the relationship of the cell form and colony form to the nature of the sugar in the medium are discussed It was noted that these yeasts fermented sucrose only after crushing the cells The normal cells fermented only glucose, fructose, mannose and maltose *Z globiformis* is especially characterized by a greater tendency to heterogamous conjugation, the formation of nonfunctioning copulation tubes, and parthenogamy This species ferments sucrose and raffinose ($\frac{1}{3}$) in addition to the sugars fermented by *Z polymorphus* Later, Krumbholz (1933) described the formation of copulation tubes without copulation in *Z globiformis* and discussed the relationship of the genus *Zygosaccharomyces* to *Torulaspora*

The small-celled osmophilic yeasts are described in detail by Krumbholz (1931b) Although no spores were found, these yeasts are referred to the genus *Saccharomyces* because of their morphological characters Lodder (1934) transfers two of the species of Kroemer and Krumbholz (1931) to the genus *Torulopsis*, as *T bacillaris* and *T stellata*, but fails to describe the third species, *T granulatus* All three of these yeasts tend to grow in clusters as small, oval to globular budding cells They ferment glucose and sucrose, but not lactose or maltose *T bacillaris* and *T stellata* also ferment raffinose ($\frac{1}{3}$)

Karamboloff and Krumbholz (1931) described another osmophilic species, *Zygosaccharomyces gracilis*, from a Portuguese wine It ferments glucose, fructose and maltose, galactose slightly, and sucrose only after the cells have been crushed It is very resistant to high acidities

The relative osmotic tolerances of a variety of yeasts are discussed by Kroemer and Krumbholz (1932) Twenty species were tested in nutrient media containing various concentrations of NaCl, $NaNO_3$, KCl, KNO_3, and glycerol The yeasts studied included a variety of common yeasts in addition to the osmophilic yeasts which the authors had previously isolated Most of the species studied showed rather high degrees of tolerance to the concentrated solutions, especially the species of *Hansenula* and *Zygosaccharomyces* Some of them grew in media containing 40 to 50 per cent glycerol Potassium nitrate was tolerated better than the other mineral salts, many of the yeasts growing in a saturated solution

Saccheti (1932) also studied yeasts in concentrated must, and isolated two species of *Zygosaccharomyces*, *Z gracilis* var *italicus* and *Z felsineus*, n s

The role of osmophilic yeasts in the fermentation of honey was studied by Fabian and Quinet (1928) They isolated 25 strains from 20 samples of honey, among them *Zygosaccharomyces japonicus*, *Z Barkeri*, and *Z priorianus* A new species, *Z mellis* (not *Z mellis acidi* Richter) is described An asporogenous yeast was named *Torula mellis* Yeasts were isolated from honey which had not fermented, as well as from fermented honey, and it was suggested that fermentation depends upon absorption of moisture by the honey from the air Thermal death points of the yeasts and their spores were determined, and pasteurization of the honey at 62 5° for 30 minutes was recommended as a preventive measure

Honey-fermenting yeasts were also studied by Marvin (1928) who found species of *Zygosaccharomyces* Wilson and Marvin (1929) found yeasts capable of fermenting 80 per cent honey on all parts of the bee, in the honey sack, in stored pollen, and on the skins of grapes Marvin, Peterson, Fred and Wilson (1931)

noted that when honey crystallizes, the water extruded from the crystallized sugar tends to dilute the solution and so reduce the sugar concentration that yeasts may grow They isolated four types of *Zygosaccharomyces*, *Z mellis*, *Z Nussbaumeri*, and two which they considered to be new species, but which were not named

Lochhead and Heron (1929) initiated a series of papers dealing with the role of yeasts in honey spoilage From 13 samples of fermented honey yeasts were isolated which not only tolerated high percentages of sugar, but failed to grow with concentrations of honey less than 32 per cent, *i e*, they are truly osmophilic rather than merely tolerant of high concentrations Four types were isolated, *Zygosaccharomyces Barkeri*, the *Z mellis* of Fabian and Quinet, *Z Nussbaumeri* and *Z Richteri* Seeking the source of these yeasts, the nectar[4] of 44 flowers was examined, and yeasts capable of growing in 80 per cent honey medium were found in all but three These fell into 11 species, of which three were *Zygosaccharomyces* species and the remainder *Torulopsis* species Sugar-tolerant yeasts were also isolated from nectar in the bee-hives, from honey tanks and from air in the extracting house The various yeasts are clearly described and illustrated Factors influencing honey fermentation, such as temperature, soluble nitrogen content, and degree of inversion of the sugar are discussed

Lochhead and Farrell (1930) searched for osmophilic yeasts in soil by inoculating soil samples into an 80 per cent honey medium No such yeasts were found in garden, orchard, clover field or cereal field soils, but were obtained from soil about apiaries, more abundantly in old ground Five species of *Zygosaccharomyces* and two of *Torulopsis* were isolated and described Lochhead and McMaster (1931) found yeasts to be present in all of 191 samples of normal honey from all parts of Canada A method of counting osmophilic yeasts in honey by serial dilutions in a 66 per cent honey medium is described

Lochhead and Farrell (1931a) counted the yeast colonies in

[4] For a discussion of non-osmophilic yeasts in nectar and a review of literature on nectar yeasts, see Zinkernagel (1929)

normal honey by plating on 60 per cent honey agar *Zygosaccharomyces Richteri* was found to be dominant in fermenting samples, but not necessarily so before fermentation begins Species of *Zygosaccharomyces* were the most frequent in occurrence in nearly all samples of normal honey *Schizosaccharomyces octosporus* was obtained from one sample

Lochhead and Farrell (1931b) found a "bioactivator" in honey which stimulated fermentation by osmophilic yeasts of the genus *Zygosaccharomyces* in synthetic media This substance is dialyzable, insoluble in ether and acetone, not precipitated by 85 per cent alcohol, non-volatile It is resistant to heat in acid solution but not in alkaline The substance is separable into two parts by adsorption with charcoal The two fractions are inert alone Farrell and Lochhead (1931) studied this "bioactivator" further in comparison with "bios" Complementary fractions from treatment with charcoal were found to exert effects similar to bios I (inositol) and bios II, when tested with the Toronto strain of *Saccharomyces cerevisiae* Charcoal treatment of honey removes by adsorption bios II leaving a residue relatively inert by itself, containing inositol Inositol, however, is not the active substance for the strain of osmophilic *Zygosaccharomyces* tested, the growth of this yeast being dependent upon the presence of another substance which, though not essential for the Toronto yeast, appears to be present in crude bios II

Lochhead (1933) concluded that not only the moisture content of the honey but also the initial yeast contamination (which may vary from one to a million per gram) are factors in determining the keeping qualities of honey With a moisture content of 17 per cent or less the honey will keep irrespective of the yeast count, with 18 to 19 per cent the honey is safe if the count does not exceed 10 per gram, with more than 20 per cent there is always danger of fermentation The content of yeast "bioactivators" did not seem to influence the keeping quality of the honey Lochhead and Farrell (1936) studied the effects of various preservatives on osmophilic yeasts In freshly extracted honey, 0 025 per cent sodium benzoate and 0 01 per cent of sodium sulphite or bisulphite prevented fermentation

In addition to the publications on sugar-tolerant yeasts in concentrated must and in honey, older literature mentions the presence of yeasts and their activities in cream candies, sugar and syrups More recently Fabian and Hall (1933) have studied yeasts causing fermentation in maple syrup They isolated a number of species *Saccharomyces aceris-sacchari* n s , *S Behrensianus*, *S monacensis*, *Zygosaccharomyces mellis*, *Z japonicus*, *Z Barkeri*, and *Z Nussbaumeri* *Saccharomyces aceris-sacchari* produces 4 spores which conjugate in pairs on germination, as in *Saccharomycodes Ludwigii*, and indeed appears to correspond very closely with *S Ludwigii* in all characters save the failure to form a cross-wall at the base of the buds *Saccharomyces Behrensianus* also forms spores which conjugate on germination, as did the strain identified as *Saccharomyces monacensis* The latter was compared with a yeast labelled *S monacensis* from Dr Tanner's collection, and found to be identical Fabian and Hall discussed the heat resistance of the yeasts they isolated, and the moisture content of the fermented syrups

Melliger (1931) studied yeasts fermenting dates, which may well be considered with the osmophilic yeasts since he found 60 per cent of sugar (invert, not sucrose) in "amhat" dates which could be exported from Egypt to Europe, "haajani" or red dates contain less sugar These dates were placed on moistened cotton in flasks until fermentation occurred, then cultures were made Curves for the rate and degree of fermentation of grape must were determined for each of the 89 yeasts isolated These curves fell into 6 groups, in particular curves for the *Saccharomyces* species could be distinguished easily from curves for the *Zygosaccharomyces* species Eight species were recognized, two of *Zygosaccharomyces*, two of *Torulopsis*, and one each of *Hanseniaspora* and *Mycoderma* The *Hanseniaspora* species was described by Lodder (1932) as a new one, *H Melligeri*

Pathogenic Yeasts

Since yeasts from many sources may lodge on the skin or be carried into the body in food or air, and since the human body in health and disease has been so thoroughly studied by bacteriolo-

gists, it is not surprising that almost all of the yeasts in the catalogue have been reported at one time or another as human parasites, often as pathogens Moreover, since yeast infections are encountered but rarely and usually reported by medical bacteriologists having but little acquaintance with this group of organisms, it is not surprising that the same species have been described under many different names It would not only take too much space, but would be almost futile to review here even the recent papers The reader interested in a complete description of all of the yeasts that have been isolated from the human body is referred to Dodge (1935)

Fortunately, with increased knowledge and a free exchange of cultures among experts, it is becoming daily more clear that the truly pathogenic yeasts of man and animals are represented by only a very few species These may be divided into two groups the yeasts which cause deep-seated infections endangering life (European blastomycosis and American "torula meningitis") caused by a yeast which has been known generally in Europe as *Cryptococcus hominis*, in America as *Torula histolytica*, and the yeasts which cause superficial infections of the skin and mucous membranes, members of the *Mycotoruloideae* and commonly referred to as the "medical Monilias "[5]

Recognition that yeasts isolated from cases of European blastomycosis and from American torula meningitis are identical apparently was made independently by Benham and by Lodder in 1934 Benham (1934) briefly reported on the identity of strains called *Cryptococcus hominis* received from Europe with strains of *Torula histolytica* from America Later, Benham (1935) compared 22 strains of this type, 12 from sources which indicated that the yeast was the primary pathogen, 7 from normal skin, 2 from feces, and 1 from a sarcoma These proved alike in morphological and cultural characters The 12 pathogenic strains produced lesions in rats, the other 10 did not except in vitamin-deficient

[5] We are, of course, including in this discussion only those pathogenic fungi which may legitimately be called yeasts, and excluding those with yeast-like growth forms in the body which grow as mycelial molds in cultures (*Blastomyces*, *Coccidioides*, *Paracoccidioides*, *Sporotrichum*, *Histoplasma*) The *Mycotoruloideae* are omitted from this review

rats She concluded that the difference in pathogenicity is one of degree only Strains from deep-seated lesions grew well at 37°, while strains from the skin surface grew poorly or not at all at this temperature Agglutination and absorption of agglutinin tests indicated complete identity of the Busse-Buschke strain of *Cryptococcus hominis* with the Freeman-Weidmann strain of *Torula histolytica* Benham recommended that all of these yeasts be designated *Cryptococcus hominis*

Lodder (1934) found the following yeasts in the Centraalbureau collection to be identical the original yeast of Busse from European blastomycosis (*Cryptococcus hominis*), the yeast (*Saccharomyces neoformans*) which Sanfelice described in 1894 as capable of producing experimental tumors in animals, the Freeman and Weidmann strain of *Torula histolytica*, a yeast isolated by Arzt from an ulcer of the tongue and named by him *Blastomyces neoformans*, a yeast isolated by Meyer in 1912 from a nasal swelling in a horse, and named by Harrison in 1928 *Torula nasalis*, a yeast isolated from a case of cutaneous "blastomycosis" by Castellani and labelled *Torulopsis hominis* var *honduriana* Since the name used by Sanfelice has priority, Lodder designated all of these pathogenic yeasts as *Torulopsis neoformans*, which she described as follows

"Cells round or short oval (4 to 6 5) μ x (4 to 7 5) μ, single or in pairs A sediment in wort, with a ring and after a long time a thin pellicle No fermentation Dextrose, levulose, mannose, galactose, saccharose and maltose may be utilized as the sole sources of carbon in synthetic media Of the nitrogen sources tested, ammonium sulphate, asparagin, urea and peptone were utilized Good growth with ethyl alcohol as the growth substrate Agar stroke cultures (75 days at 15°C) yellowish, soft, moist, shiny, mucoid, smooth, with a smooth border "

The reviewer would like to add to this description the occurrence of a light tan color with a slightly rosy cast on 5 per cent dextrose, 1 per cent Bacto-peptone agar Such a color may appear in old cultures of other yeasts, but has been present after a few days in all of the strains of *Torulopsis neoformans* which I have studied Another important diagnostic character is the development of voluminous capsules about the yeast in infected tissues

A further step in establishing the identity of this yeast was made by Todd and Herrmann (1936) who described the formation of ascospores Studying two strains (one isolated at the University of Iowa, the other from Minnesota) from cases of torula meningitis, they observed spore formation in cultures aged until the medium began to dry (3 to 6 weeks) using Sabouraud's glucose or maltose agar The single spores and their asci are somewhat different from those found in any other described yeasts The spore is contained within a cell with greatly thickened walls, and is excentric in position The ascus may become heart-shaped, with the spore occupying the apex of the heart, from which it is discharged at maturity They noted the development of two sorts of cells in the aging cultures, a small thin-walled cell, and a large thick-walled cell, and observed the fusion of these in hanging drop cultures A life cycle is described and illustrated, rather complicated in that the ascospore appears to bud off several cells before it finally throws off the "hull" of the ascus Since the yeasts they studied formed single spores by heterogamous conjugation they referred them to the genus *Debaryomyces* and suggested that the sporogenous pathogenic yeasts be called *Debaryomyces hominis*

I had no difficulty in verifying the observations of Todd and Herrmann with the strain which I had furnished them, and with another strain isolated later from a case of "torula meningitis" No such structures appeared, however, in a yeast otherwise identical isolated from a myxoma-like lesion of the thigh Todd and Herrmann reported finding spores in 8 other strains, including the Freeman-Weidmann strain which had been studied by Benham and by Lodder

The observations of Todd and Herrmann were confirmed by Redaelli, Ciferri and Giordano (1937) They studied 19 strains from cases of meningitis and encephalitis (all originally labelled *Torula histolytica*), Sanfelice's original strain of *Cryptococcus neoformans*, the *Cryptococcus psichrophilicus* of Nino, and the *Torula nasalis* of Harrison With all of these the formation of single spores was observed essentially as described by Todd and Herrmann, except that conjugation appeared at times to be

isogamous as well as heterogamous The formation and liberation of buds by the ascospore while still within the ascus they consider to be a procedure for extruding excess fat ("exosmose par blastospores") The Italian authors also noted with some strains a warty wall characteristic of the spores of *Debaryomyces* Taking recognition of the synonymy previously established by Benham and by Lodder, they propose *Debaryomyces neoformans* as the proper scientific name for these pathogenic yeasts, and this appears to me to be the valid name for most of them

This yeast was studied further *in extenso* by Giordano (1938) from the standpoint of taxonomy, physiology and pathogenicity Some 29 strains were examined Sixty-eight synonyms are listed! The formation of ascospores is reconfirmed Giordano points out the analogy between the pathogenic yeasts in question, and the apiculate yeasts, in both groups some strains form spores, while other strains otherwise identical do not Are they to be listed in separate genera? Giordano believes that a careful study will show fewer and fewer asporogenous strains A large part of this paper is devoted to a study of the lesions in experimentally inoculated animals

Of the yeasts described as pathogenic which do not fall in synonymy with *Debaryomyces neoformans*, nearly all may be dismissed (excepting the "medical Monilias") as lacking proof of pathogenicity They have been isolated from superficial lesions where they may well have been simple contaminants, and are not pathogenic to laboratory animals One further species, however, deserves some consideration This is a yeast first isolated by Anderson in 1917 from feces and named by him *Cryptococcus glabratus* It was reisolated from the intestines by Benham (1935) who not only found identical cultural characters but also complete reciprocal absorption of agglutinins with Anderson's strain It was nonpathogenic for rabbits Black and Fisher (1937) reported isolating this yeast (in large numbers) from the nasopharynx of a boy suffering from bronchopneumonia, the cultures were made to obtain pneumococci for typing, since no sputum was obtained Their strain did not kill rats, but produced lesions in the omentum from which the yeast was reisolated

Lodder and de Vries (1939) studied five strains of this yeast Anderson's original strain, one which had been described by Ota in 1924 and was derived originally from the Dermatologic Clinic at Bern, one from an ulcer of the vulva, one from urine of a diabetic, and finally one from sputum in a patient suspected of tuberculosis It should be noted that the patient with the ulcer of the vulva gave a positive skin test to this yeast while other persons gave negative reactions, and that the yeast was isolated from the sputum of the pulmonary case in large numbers on three occasions over six months, and that in spite of extensive x-ray findings no tubercle bacilli were found Five rats were inoculated intracardially with each of the five strains Of these 25 rats, five died after some months, with pleural lesions, and the yeasts were reisolated from the lungs This yeast therefore appears to be of frequent occurrence in man and may be pathogenic It should be kept in mind by those who have occasion to study yeast infections Lodder proposed changing the name to *Torulopsis glabrata* It differs from *Debaryomyces neoformans* especially in its ability to ferment dextrose, levulose and mannose with the production of gas, galactose, sucrose, maltose and lactose are not fermented Alcohol is not utilized

Pityrosporum

In spite of the fact that this organism has been known since 1874 to be commonly present in the skin, especially of the scalp, and thought by some to be the cause of seborrhoeic dermatitis, it remains somewhat of a mystery Commonly called the "bottle bacillus" it is said to have been named *Pityrosporum Malassezi* by Sabouraud in 1895, but according to Lodder (1934) the original publication in which this name was given cannot be found A number of microorganisms have been cultivated from the skin and have been said to be the true *Pityrosporum*, but they differ among themselves Lodder (1934) describes three species in the Centraalbureau collection *P Malassezi*, isolated by Benedek from a case of pityriasis capitis, *P pachydermatis* isolated by Weidman from the skin of a rhinoceros, and *P rhinoserum* originally from Sabouraud's laboratory The latter was found to be identical with *P pachydermatis*

The organism which Sabouraud is said to have named *Pityrosporum Malassezi* is most frequently referred to as *Pityrosporum ovale* since it is thought to be identical with one which had previously been described by Bizzozzero as *Saccharomyces ovalis*

The organism isolated by Benedek (1930) from dandruff scales in maltose broth hanging-drop cultures is described by Lodder (1934) as follows "Growth very slow Cells short-oval, oval, or flask-shaped (2 5 to 3 8) μ x (4 to 5 5) μ, single or in pairs or rarely in threes Budding frequently on a broad base In wort no ring or sediment No fermentation No growth on ethyl alcohol medium Wort-agar stroke culture hardly visible, matte, not differentiated in color from the agar"

Ota and Huang (1933) isolated an organism which may be identical with that of Benedek Scales were soaked in 60 per cent alcohol and after washing in the condensation water, placed on the surface of agar slants containing butter or lecithin in the medium

Moore (1935) rejected the organism described by Benedek because it is too large The organism as it occurs in dandruff scales was given dimensions of (0 8 to 1 5) μ x (2 to 3) μ by Benham (1939) Moore made cultures on Difco wort agar A variety of common molds grew as well as the yeast which he describes The latter varied considerably in morphology, and the morphology was different on different media The growth on agar was pinkish to buff Cultural characters on a variety of media are described Acid, no gas, was formed from galactose, dextrose, d-mannose, levulose, maltose, sucrose and melitose

Moore, Kile, Engman and Engman (1936) reported further on the organism which Moore had isolated They note that the organism is also variable in morphology in the scales of epidermis according to the acuteness or chronicity of the disease Inoculation by scarification gave reactions in patients, rabbits and guinea pigs

Benham (1939) studied cultures isolated by Moore and found them to fall in her group III of "Cryptococci" which we have noted previously appears to be identical with *Debaryomyces neoformans* The organism of Moore fell in the nonpathogenic subgroup, not growing at 37° Benham isolated a variety of yeasts

from seborrhoeic scalps on wort agar, among them a very small oval one which failed at first to grow on subcultures When ether washings of the scalp and ether extracts of comedones were added to the medium, growth continued With this clue a variety of fatty materials were studied Eight strains of the yeast were isolated from 30 cases Although on ordinary wort agar growth is slight and transparent, as described by Lodder, a rich growth is obtained on media containing fatty materials Lanolin, butter and stearic acid were especially favorable, pork fat, chicken fat and linseed oil gave a lesser growth, while a number of other oily substances gave only poor or no growth The cells are small (2 to 3) μ x (4 to 5) μ, often flask-shaped, the buds with a broad base, often separated from the parent cell by a cross wall as in *Saccharomycodes* or *Schizoblastosporion*

Emmons (1940) found that an organism identical with that of Benham and of Ota and Huang could be readily isolated in dextrose broth containing glycerol Bacterial growth is entirely inhibited at 28 per cent glycerol, while *P ovale* continues growth up to 48 per cent In these primary cultures the organism grows on nutrients provided by the epidermal scales Subcultures would not grow in any concentration of the glycerol-broth Subcultures grew readily on acid dextrose agar over which an ether extract of lanolin or oleic acid had been spread Morphologically and culturally the strains isolated by Emmons appeared to be the same as those described by Benham, not at all like those isolated by Moore

It appears that the organisms isolated by Benedek, Ota and Huang, Benham and Emmons are sufficiently alike to be considered identical, and to be so like the organism that is found in dandruff scales that they may be considered to be the true *Pityrosporum ovale* Whether this organism is the cause of seborrhoeic dermatitis is, however, doubtful The disease itself is so vague and variable as seriously to limit critical experimentation Apparently identical lesions have been produced by scarification inoculation of widely different organisms *Pityrosporum* may be found on normal skin, especially in the sebaceous glands

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THE CYTOLOGY OF BACTERIA

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CONTENTS

It is quite impossible to include all of the literature of this subject in the space available here Accordingly, this review is limited to the publications dealing with non-protoplasmic cell inclusions, the problem of the nucleus, the cytology of reproductive bodies, the cell wall and cytoplasmic membrane and the flagella The last comprehensive review, "Die Zelle der Bakterien" by Arthur Meyer, was published in 1912 Since that time new methods of investigation have been developed, and many additional papers have appeared A brief review by Knaysi (86) includes much of the more recent literature

It must be understood at the outset that the subjects to be

treated have long been the cause of much controversy and that many conflicting reports have been published The subject is difficult, and while it is obvious that many errors have been made in the past, there is probably no one who is fully competent to evaluate all the conflicting reports My aim has been to present both sides of controversial matters as impartially as possible and to draw such conclusions as the evidence appears to warrant

According to modern cytological terminology, a plant cell consists essentially of a nucleated mass of cytoplasm, the protoplast, surrounded by a cell wall The surface of the protoplast is a semipermeable cytoplasmic membrane Additional structures such as plastids, non-protoplasmic cell inclusion bodies, centrosomes, blepharoplasts and chondriosomes may occur in some cells With some reservations concerning the presence of a visible nucleus, we may assume that the bacterial body is a cell, comparable to the cells of other fungi Accordingly, the terms bacterial cell, protoplast, cytoplasmic membrane, cell wall, and cell inclusion bodies will be employed throughout this review

I. CELL INCLUSIONS

The term cell inclusions is quite generally employed by cytologists to denote certain non-protoplasmic bodies which are deposited in the protoplast Such bodies occur in cells of higher green plants, algae, yeasts, higher fungi and bacteria They are generally absent from young actively growing cells but are formed *de novo* as the cells become older Sap vacuoles, fat droplets, grains of volutin, starch, aleurone, glycogen, iogen, sulphur, and mineral crystals are appropriately spoken of as cell inclusions Inclusion bodies may occur within the sap vacuoles or they may be embedded in the cytoplasm

The occurrence of granules in bacterial cells has been known since the early work of Koch (90) who observed highly refractile bodies in unstained cells of *Mycobacterium tuberculosis* Babes (10) and Ernst (41, 42) reported stainable granules in various species of bacteria Many papers have since been published and almost every conceivable opinion has been expressed concerning the nature, origin and biological significance of bacterial granules

They have been identified as spores, sporoids, spore primordia, gonidia, gametes, nuclei, chromatin granules, and non-protoplasmic inclusions

Fischer (45) took the extreme position that all granules of bacteria are cell inclusions consisting of reserve materials Although this view has been questioned by many investigators, there are very good reasons to believe that many of the bodies which have been described as nuclei, chromidia, and gonidia were nothing but non-protoplasmic cell inclusions

Our knowledge of bacterial cell inclusions had its beginning in the work of Meyer (116) who introduced accurate microchemical methods of study We now know that many bacteria contain fat bodies, grains of volutin, glycogen, iogen and sap vacuoles Sulphur and mineral crystals are found in the sulphur bacteria We may now consider the various inclusions in detail

1 *Fat bodies* Fat occurs in some bacteria as highly refractile, spherical, oval, or elongated bodies which resemble endospores and were erroneously identified as spores by some early investigators The bodies are variable in size and form, but are generally spherical They may reach a diameter equal to that of the cell or may even extend the dimensions of the cell body They are usually lined up in a row in the median axis but may be scattered throughout the cytoplasm When numerous, they become somewhat flattened and limit the cytoplasm to thin lamellae

Fat bodies are not stainable by any of the methods usually employed for staining bacterial cells They appear, therefore, as clear spaces in cells stained with aqueous solutions of aniline dyes and for this reason have been mistaken, at times, for endospores or vacuoles Various methods are now known by which fat droplets may be stained selectively Meyer (117) employed Sudan III, alkanin, and dimethylamidoazobenzol, but more satisfactory dyes are now known Other dyes of the Sudan series have been found satisfactory for staining bacterial fat The new compound Sudan black B, was tested by Hartman (66) and proved to be greatly superior to the earlier dyes This new dye, when dissolved in pure ethylene glycol, is stable indefinitely, and is almost

free from the troublesome precipitates which are unavoidable in some methods of fat staining It stains the fat droplets of bacteria and yeasts quickly, intensely and selectively

The naphthol blue method discovered by Dietrich and Liebermeister (31) and modified by others (21, 38, 120, 164), has been employed extensively for staining fat bodies in bacteria, yeasts, and fungi Eisenberg (38) found that certain aniline dyes, which stain the cell membrane and cytoplasm but not the fat bodies, may be treated with various precipitating agents to obtain useful fat dyes When cells containing fat bodies are placed in a dilute aqueous solution of Nile blue sulphate, the bodies remain unstained, but upon addition of alkali, an orange-red precipitate is formed and the fat bodies are stained selectively Similarly, the precipitates formed by treating an aqueous solution of basic fuchsin with iodine, picric acid, alkaline phenol, or alkaline α-naphthol may be used as fat dyes Although the precipitates are soluble in alcohol and may be employed as dilute alcoholic solutions, better results are obtained by suspending the cells in the precipitating agent and adding an equal volume of dilute basic fuchsin The staining action appears to depend on lipoidal solubility of the precipitates, they stain fat bodies by dissolving in them and saturating them with the dye The same is true of indophenol blue, the dye synthesized in Dietrich and Liebermeister's method It should be noted that dried fixed films are not suitable for any of these fat staining methods

The species of bacteria which deposit fat are shown in table 1 The list is probably incomplete and there may have been some erroneous reports The record shows that fat bodies occur in some species of *Bacillus* but not in others, that all species of *Spirillum*, *Azotobacter* and *Rhizobium* have been found positive, and that fat bodies have not been reported in the corynebacteria, clostridia or any of the coccaceae

There is still some controversy concerning the occurrence of fat bodies in mycobacteria Dorset (36) reported successful staining of tubercle bacilli with Sudan III and thought he had discovered a useful method for diagnosis He described the stained cells as beaded rods However, LeDoux (94) could not

TABLE 1

The occurrence of glycogen, fat and volutin in certain bacterial species

SPECIES	GLYCOGEN	FAT	VOLUTIN	REFERENCE
Azotobacter beijerinckii	–	+	+	98
Azotobacter chroococcum	–	+	+	98
Bacillus alvei	–	–	+	131
Bacillus anthracis	–	+	–	53
Bacillus asterosporus	+	–	+	117
Bacillus carotarum	+	–	–	50
Bacillus cohaerens	+	–	–	52
Bacillus ellenbachensis	–	+	+	131
Bacillus fusiformis	–	–	+	131
Bacillus graveolens	–	+	–	131
Bacillus lacticola	–	+	+	131
Bacillus lactis	–	+	+	131
Bacillus megatherium	–	+	–	131
Bacillus mycoides	–	+	–	50
Bacillus oxalaticus	–	+	–	131
Bacillus parvus	+	–	–	131
Bacillus petasites	–	+	–	50
Bacillus pumulus	+	–	–	131
Bacillus robur	+	+	+	131
Bacillus ruminatus	–	+	–	50
Bacillus silvaticus	–	+	–	131
Bacillus simplex	+	–	–	50
Bacillus sphaericus	–	–	+	131
Bacillus subtilis	+	–	–	50
Bacillus teres	+	–	–	131
Bacillus tumescens	–	+	–	117
Bacterium globiforme	–	–	+	126
Clostridium butyricum	+	–	–	117
Corynebacterium diphtheriae	–	–	+	10
Corynebacterium fimi	–	–	+	126
Corynebacterium hoagii	–	–	+	126
Corynebacterium simplex	–	–	+	126
Corynebacterium tumescens	–	–	+	126
Corynebacterium ulcerans	–	–	+	126
Corynebacterium xerose	–	–	+	41
Lactobacillus bulgaricus B	–	–	+	193
Mycobacterium leprae	–	–	+	12
Mycobacterium phlei	–	+	–	52
Mycobacterium tuberculosis	–	+	–	52
Pseudomonas sp ?	–	–	+	52
Rhizobium japonicum	–	+	–	99
Rhizobium leguminosarum	–	+	–	99
Rhizobium lupini	–	+	–	99
Rhizobium meliloti	–	+	–	99
Rhizobium trifolii	–	+	–	99
Sarcina ureae	–	–	–	39
Spirillum giganteum	–	+	+	39
Spirillum serpens	–	+	+	100
Spirillum tenue	–	+	+	100
Spirillum undula	–	+	+	100
Spirillum virginianum	–	+	+	100
Spirillum volutans	–	+	+	100
Streptococcus tirogenus	–	–	–	39

obtain satisfactory staining although he followed the same method Dorset was not able to obtain positive results with several lots of dye and concluded that success depends on some unknown quality of the dye compound

Grimme (52) stained the refractile granules of *Mycobacterium phlei* and *M tuberculosis* with Sudan III and dimethylamidoazobenzol and identified them, accordingly, as fat bodies Meyer (129) confirmed Grimme's results and expressed the opinion that most of the fat extractable from tubercle bacilli is located in the bodies rather than in a fatty membrane, as had been generally believed Knaysi (83) saw stained granules in cells of tubercle bacilli which had been treated with Sudan III He found, however, that the granules remained stainable after treatment with fat solvents and denied their fatty nature Hartman (66) stained the refractile granules of various mycobacteria with Sudan black B and identified them as fat bodies

Although some workers have reported negative results, there is a preponderance of evidence that mycobacteria deposit fat in the form of definite bodies When due allowance is made for differences in the quality of dyes and improper technique, there is little or no conflicting evidence

The biological significance of fat bodies in bacteria appears to be the same as in other organisms They probably function as reserve food in some cases while in others they may denote fatty degeneration of the cells Grimme (52), Preisz (153), Meyer (123), Lewis (97) and others have shown that the fat bodies disappear when spore formation occurs and when cells are placed on agar devoid of nutrients There is some evidence for the theory of fatty degeneration Muller and Stapp (129) and Almon (5) proved that granulated bacteroids from root nodules are not viable Fat formation in *Endomyces vernalis* has been described recently by Heide (67) who supports the theory that the fat bodies function as reserve food

The conditions necessary for deposition in the form of definite visible droplets do not appear to be very well understood Many species of bacteria which do not form visible droplets may contain relatively large amounts of extractable fats This phase of

the subject has been reviewed by Meyer (123) and by Buchanan and Fulmer (22).

The occurrence of fat bodies in bacteria has been the cause of many erroneous interpretations of cell structure and methods of reproduction. Various workers, failing to recognize the true nature of the bodies, have identified them as endospores (90), spore primordia (23), sporoids (160), endoplasts (135), and non-stainable gonidia (48, 104).

Cells containing fat bodies do not stain uniformly when treated with protoplasmic dyes but present an appearance which has been frequently described as interrupted, speckled, granulated, barred, beaded, banded, vacuolated and alveolar. The writer (98-100) has shown that uneven staining in cells of species of *Azotobacter*, *Rhizobium*, and *Spirillum* is conditioned by non-stainable fat bodies embedded in the stained cytoplasm. The stainable compressed cytoplasm has been regarded by some investigators as chromatin, by others as reproductive bodies.

2. *Volutin.* Refractile granules, variously designated in the literature as Babes-Ernst granules, metachromatic corpuscles, and volutin grains, occur in many species of bacteria, yeasts, molds, higher fungi, and algae. According to Meyer (121), they are not found in any group of plants above the *Thallophyta*. Guilliermond (58) questioned this conclusion but offered no convincing proof to the contrary. Such granules have been known in bacteria since the early work of Ernst (1888) and Babes (1889). The latter (11) observed red granules in cells of bacteria which had been stained with methylene blue. He introduced the term metachromatic corpuscles to designate this peculiar staining reaction. The term volutin, now almost universally employed, was proposed by Grimme (52) to designate the stainable granules of *Spirillum volutans*.

Volutin is a viscous substance which may occur in the form of tiny droplets, large globoids, irregular bodies or elongated threads (121). The bodies are somewhat more refractile than cytoplasm, but they are less refractile than fat bodies and spores. The principal tests by which volutin is distinguished from other cell inclusions and protoplasmic structures were given by Grimme

(52) and Meyer (121). These granules dissolve and disappear from cells in water at 80° within 5 minutes, and still more quickly in boiling water. They are readily soluble in strong or dilute solutions of alkalies, in 5 per cent sulphuric acid, fresh Javelle water, and chloral hydrate, but not in picric acid, ether, chloroform, alcohol, trypsin, pepsin or 1.0 per cent sulphuric acid. When the cells are fixed by formaldehyde, osmic acid, alcohol, or the usual method of heating dried films, the granules resist solvents.

Volutin grains stain more intensely than cytoplasm with basic aniline dyes, but they do not stain with fat dyes. Heucke and Henneberg (71) have shown that neutral red, 0.001 to 0.005 per cent in water, stains the bodies *intra vitam* but has no staining capacity for cytoplasm. Similarly, dilute aqueous solution of methylene blue causes intense staining of the granules with little or no action on cytoplasm. They are markedly resistant to the destaining action of 1 per cent sulphuric, hydrochloric or acetic acid. Differential staining of the granules and cytoplasm may be accomplished by staining with methylene blue, destaining with 1.0 per cent sulphuric acid and counterstaining with a contrast dye. The replacement dye, vesuvin, may be employed as in Ernst's first method (41) to effect differential staining of granules and cytoplasm. He stained fixed films with methylene blue and, after rinsing with water, counterstained them with vesuvin. The deep blue granules stand out sharply in the yellowish cell body. Safranin may be substituted for vesuvin. Contrast staining is attained also by treating fixed stained films with methylene blue and Lugol's iodine solution which causes blackening of the volutin bodies.

Various special methods have been devised for staining the granules of diphtheria bacilli and have been widely used for diagnostic purposes. The methods of Neisser (133), Albrecht (2), and Albert (1), have been found useful.

The biological significance of volutin was not well understood by the earliest investigators, and almost every conceivable function was assigned to it. Ernst (41) spoke of the deeply stained bodies as spores. Neisser (132) reported acid-fastness and re-

garded the granules as true spores. Ernst (42) reversed his former opinion and introduced the term sporogenic granules. According to his conception, the granules consist of nuclear material and participate directly in the formation of spores by fusing together. This theory of spore formation has been widely held by many subsequent investigators, but there is no reason to believe that volutin bodies consist of nuclear material or that they participate in the formation of spores by fusion. Babes (10) saw no analogy between the granules and true spores. He seems to have reached no very definite opinion concerning their function, but suggested a possible relation to cell division and spore formation. Marx and Woithe (110) favored the idea that the granules function in cell division but opposed Ernst's theory of spore formation. They spoke in favor of a correlation between virulence and the presence of granules in pathogenic species. This theory of toxigenic granules was attacked and discredited by Krompecher (92), and others (44, 47). Volutin has been frequently confused with nuclei, chromidia, compressed cytoplasm, spore primordia and gonidia.

The true significance of volutin in bacteria appears to have been first recognized by Grimme (52) who spoke of it as a reserve food compound. Guilliermond (58) reached the same conclusion concerning the function of volutin in yeasts and other fungi. Meyer (121), who had previously discussed the function of fat, glycogen, and iogen in bacteria, supported Grimme's explanation.

It is now well known that volutin grains are not permanent organs of the cell, they arise *de novo* in cells of some species but never occur in others. The deposition of volutin in bacteria depends on the species, the age of the cells, and to some extent on the culture medium. They are not found in very young actively growing cells but may become abundant as the cells mature. Zikes (202) showed that the culture medium must contain phosphate. In spore-forming species, the granules reach their greatest abundance just preceding spore formation and disappear during the ripening of the spore. For these reasons, volutin must be considered as a storage product which functions as reserve food.

The chemistry of volutin has not been studied very extensively Meyer (121) noted marked similarity between some microchemical reactions of volutin grains and nucleic acid extracted from yeast He concluded that the substance is a nucleic acid compound which differs from the nucleoprotein found in the chromatin of nuclei He reasoned logically that volutin from various species might differ in precise composition in much the same manner as fats differ According to Schumacher (166), free nucleic acid becomes green when treated with methylene blue and chrysanilin while nucleoproteins become yellow Since the granules of diphtheria bacilli take the green color when stained by this method, they should consist of free nucleic acid Glaubitz (49) was not able to obtain a positive Schumacher test for nucleic acid in the volutin grains of yeast Zikes (202) studied the chemistry of volutin by macrochemical methods and identified it as a nucleoprotein Although the chemical nature of volutin may not be fully known, it can be identified by a series of microchemical tests which serve to distinguish it from all other cell inclusions and protoplasmic structures

The bacterial species which deposit grains of volutin are shown in table 1 It is by no means probable that the list is complete or that all the original determinations were correct Prior to the studies by Grimme and Meyer, methods of identification were not very reliable and errors are to be expected It appears from the record that volutin occurs in all species of *Spirillum* and *Azotobacter*, in some species of *Bacillus*, *Aerobacillus*, *Lactobacillus*, *Corynebacterium*, *Pseudomonas*, and *Mycobacterium*, but not in *Rhizobium* and *Sarcina*

The writer doubts the occurrence of volutin in mycobacteria although Babes (12) and Guilliermond (59) reported its presence in *Mycobacterium tuberculosis* Hollande and Crémieux (75, 76) also described granules which resembled volutin but which differed from it in some respects Similarly, Knaysi (83) saw hyperchromatic granules which were not soluble in hot water According to my observation volutin does not occur in any of several species examined Stainable granules have been reported in various species of cocci but no very critical investigation concerning their identity has been made

We are not specifically concerned with the occurrence of volutin in other groups of microorganisms but may call attention to its wide distribution in yeasts, actinomycetes, molds and higher fungi It appears to be present in all species of *Cyanophyceae* where it is deposited abundantly in the central body which resembles the large sap vacuole of yeasts It has been reported in various protozoa by several investigators This wide distribution of the same substance in various microörganisms seems to indicate an important reserve material which functions in the same manner as fats, glycogen, starch and other storage compounds

In the study of the bacterial cell, we are especially concerned with volutin as a cause of error in the interpretation of cell structure and methods of reproduction This subject will be discussed in detail in a subsequent section

3 *Glycogen* Glycogen is a reserve or storage carbohydrate which occurs commonly in the cells of animal tissues, yeasts, molds, and some species of bacteria According to Meyer (123), glycogen usually occurs in the form of viscous masses which are somewhat refractile Microchemical tests for glycogen are less satisfactory than for other cell inclusions The masses become reddish brown in strong Lugol's iodine solution and are, therefore, distinguished from the yellowish cytoplasm

Glycogen has been reported in several species of *Bacillus* and in *Clostridium butyricum* as shown in table 1

The refractile granules of *Azotobacter* were identified erroneously as glycogen by Heinze (68) Muller and Stapp (129) were not able to confirm this report by precise macrochemical methods of study Similarly, Hiltner (72) reported glycogen in bacteroids of some rhizobia, but the identification was not confirmed (99, 171)

4 *Iogen* Small refractile granules which stain blue with iodine have been known since the early work of Trécul (1865) Beijerinck (16) employed the term granulose which is still preferred by many bacteriologists Meyer (117, 123) included all carbohydrates of bacteria under the terms iogen and glycogen and distinguished between them by differences in the reaction with iodine Gray (51) identified as starch grains the granules of *Escherichia coli* which stain blue with iodine Svartz ((179) and in earlier papers) studied the iodophilic granules of intestinal clostri-

dia The occurence of this compound in various species, especially in the genus *Clostridium*, has been established Iogen appears to serve as reserve food

II. THE NUCLEUS

The question of the presence or absence of a bacterial nucleus, its nature, and the forms which it may assume, if present, has long been a subject of interest to cytologists, but no wholly satisfactory answer has yet been found Many of the earlier workers assumed that the bacteria, standing as they do at the threshold of organized living matter, may lack some of the characteristic structures which occur normally in the cells of higher plants and animals Haeckel introduced the term cytode to denote such cell-like organisms This doctrine was acceptable to Fischer (45), Migula (124) and other early bacteriologists who regarded bacteria as non-nucleated organisms The voluminous literature which has appeared during the past fifty years shows, however, that the theory has not been generally accepted Henrici (69) said "It satisfies the facts as we know them, but it does not satisfy the mind " There are some cytologists, perhaps many, who would challenge the statement, that the theory of no nucleus satisfies the known facts, but there is no doubt that it fails to satisfy the mind Theoretically, it seems, we should expect to find a true nucleus or a functional equivalent in the cells of bacteria

The literature of the subject is truly voluminous, frequently contradictory, and highly controversial, it is impossible to reconcile the numerous conflicting reports and theories which have been based on the study of *Bacillus anthracis* alone This much, however, is certain cell inclusions, immature spores, and cytoplasmic structures have been frequently mistaken for nuclei, in some cases the organisms studied were not true bacteria, and the methods employed were not always suitable for cytological study It is also true that some investigators, inadequately trained and without previous cytological experience, were not properly prepared for such a difficult undertaking For all of these reasons, much of the old uncritical work is of little value, and need not concern us here

We may conveniently divide the theories concerning the nucleus of bacteria into the following groups

1 The bacteria do not possess a nucleus or its equivalent
2 The cell is differentiated into a chromatin-containing central body and peripheral cytoplasm
3 The bacterial body is a nucleus devoid of cytoplasm a naked nucleus or nuclear cell
4 The nucleus consists of several chromatin bodies, a chromidial system, scattered throughout the cytoplasm
5 The form of the nucleus is not constant throughout the growth cycle, it may occur as a discrete spherical body, an elongated chromatin thread, or scattered chromidia depending on the stage of development a polymorphic nucleus
6 The nuclear substance consists of fine particles of chromatin dispersed uniformly in the cytoplasm but is not distinguishable as morphological units a diffuse nucleus
7 The protoplast contains one or more true vesicular nuclei
8 The nucleus is a naked invisible gene string, or a chromatin-encrusted gene string analogous to a single chromosome

1 *No nucleus or equivalent* Among the early workers, Fischer (45) was the most able advocate of the theory that a bacterial cell possesses no nucleus He wrote as follows "The bacterial cell then, interpreted in the light of the above facts is a simple protoplast enclosed within a cell-membrane but devoid of a nucleus" In another connection, he said "The general conclusion to which all these observations lead us is that the bacterial cell-contents are a mass of protoplasm representing an osmotic system precisely like that of the cells of higher plants, but, unlike them, having no nucleus" He saw granules in various species of bacteria, but he regarded them as reserve inclusion bodies rather than nuclei Migula (124) appears to have reached similar conclusions although he expressed his views in somewhat guarded terms Wamoscher (191) studied several species by micrurgical methods, and saw nothing which could be interpreted as a nucleus Roman (158) found no nucleus in *Mycobacterium tuberculosis* Alexieff (3) denied the occurrence of chromatin in the cells of bacteria and *Cyanophyceae*

2 *The central body* Butschli (25) advocated the theory that the bacterial cell, like the cells of *Cyanophyceae*, is differentiated into a dense nucleus-like organ, the central body, and a peripheral layer of cytoplasm He saw complete analogy in the structure of *Spirillum volutans* and a species of *Oscillatoria* although, according to his observation, the cytoplasm in *S volutans* is reduced to a thin outer layer and two polar caps from which the flagella arise The occurrence of deeply stainable chromosome-like bodies in the central portion of bacterial and cyanophycean cells gave him complete assurance that the central body is the homologue of a true nucleus

The nature of the central body, a prominent vacuole-like structure which occurs in all species of *Cyanophyceae*, has long been a controversial matter among botanists This cell organ resembles a true nucleus in certain characteristics it occurs in every cell as a definite, relatively large, spherical body, it contains chromosome-like granules, and it divides simultaneously with division of the cell For these reasons, many botanists have regarded it as a primitive nucleus which differs from a true nucleus in one important respect, namely, the absence of a nuclear membrane Others, notably Meyer (121), objected to this interpretation According to Meyer, the central body is not a nucleus but a large central sap vacuole which contains volutin and is, therefore, comparable to the vacuole of yeasts More recently, Hollande (73) described deeply stainable granules, *nucleosomes*, which occur among the volutin grains According to his interpretation, the "nucleosome apparatus" is a protoplasmic secretion Whatever may be the final outcome of the controversy concerning the central body in the *Cyanophyceae*, evidence is not sufficient to warrant the assumption that an homologous cell organ occurs in any of the true bacteria

Zettnow, in his early writings (197-200), described the bacterial cell as a structure which consists essentially of a central chromatin-containing portion, the endoplasm, surrounded by a thin envelope, the ectoplasm In large spirilla and in some bacillary species, he saw well-differentiated chromatin bodies, but in various species of small bacteria the chromatin appeared to be

more finely divided and diffused in the endoplasm Later, Zettnow (201) concluded that the bodies he had formerly called chromidia were grains of volutin and that chromatin, if present, is in the form of minute invisible particles uniformly dispersed in the cytoplasm The terms endoplasm and ectoplasm are still employed by some bacteriologists

Petit (141) found volutin bodies in the cells of *Chromatium okenii* and other sulphur bacteria but no true nucleus or central body A central body was present in *Oscillospira guilliermondi*, but he regarded this species as an alga Guilliermond (60) reached similar conclusions concerning *Thiodictyon* Some support was given to the theory of a central body in true bacteria by Guilliermond (61) who studied *Bacillus megatherium* and observed a central organ resembling that of the *Cyanophyceae* According to Hollande (74) the structure described by Guilliermond is a metanucleosome Lewis (100) studied the structure of *Spirillum volutans* and other spirilla No support was found for the theory of a central body

3 *A naked nucleus* The fact that the staining capacity of bacteria is similar to that of the nucleus of ordinary cells has caused some investigators to regard the whole body as the homologue of a true nucleus This theory has been adversely criticised as well as supported on theoretical grounds alone The hypothesis has been put forward that the first living things to arise on the earth consisted of undifferentiated protoplasm similar to the substance of true nuclei, and that cytoplasm is the result of subsequent evolution The opponents of this idea have contended that the most primitive living things consisted of undifferentiated protoplasm homologous with the cytoplasm of higher organisms, and that differentiation into cytoplasm and nucleus represents the final result of evolutionary development According to these hypotheses, there could be a variety of cell or cell-like organisms with different degrees of differentiation true cells with typical nucleus and cytoplasm, cell-like bodies devoid of a nucleus or devoid of cytoplasm, and cells differentiated into cytoplasm and a primitive nucleus

Aside from similarity in stainability, there is no substantial

evidence to support the theory that bacteria are naked nuclei Růžička (159, 160) attempted to prove that the bacterial body is identical morphologically with true nuclei of other plants His claims were based principally on the structure of *Bacillus anthracis* when cultivated on glycerol agar The cells failed to form endospores on this medium, when stained, they simulated the structure of true nuclei He described non-stainable masses of linin and net-like stainable chromatin His drawings bear a superficial resemblance to diagrammatic drawings of true nuclei, but there is no reason to believe that the structures involved are related in any way Růžička's work was severely criticized by Eisenberg (38), Meyer (123), and others According to Meyer, the cells which failed to form endospores became filled with non-stainable fat bodies which were incorrectly designated as linin while the chromatin net was nothing but compressed masses of cytoplasm lying between the fat bodies The criticism appears to accord with all known facts

Some support for the theory was afforded by Ambroz (8), a student of Růžička's, who studied *Bacillus nitri* but was unable to add anything of value More recently, Kŭzela (93) studied the structure of *Bacillus anthracis* and various other species of bacteria by means of the Feulgen-Rossenbeck reaction Support was found for the analogy between the bacterial cell and the nucleus of ordinary cells

The hypothesis that the most primitive form of living things must have consisted of undifferentiated substance, analogous to that of true nuclei, appears to be logical enough since we know that the nucleus is the bearer of hereditary units and directs the activities of the cell However, there is no evidence that bacteria are representatives of such a theoretical primitive organism On the other hand, all the evidence indicates that the bacterial body possesses most, if not all, of the essential features of a true cell We are not concerned here in a theoretical discussion of living things still more primitive than the known bacteria, although it is not denied that such organisms may have existed or may still exist

4 *A chromidial system* The theory that the bacterial nucleus consists of visible chromatin bodies, a chromidial system, scat-

tered throughout the cytoplasm has been advocated by many cytologists but has not gone unchallenged The theory seems to have had its inception in the early work of Ernst (42) who observed stainable bodies in various species of bacteria Support was given by R Hertwig (70), who observed scattered chromidia in the cytoplasm of certain nucleated protozoan cells Reasoning by analogy, he proposed that the nucleus of the bacteria and *Cyanophyceae* consists of a chromidial system His exact language is of interest for it marked the beginning of a theory which attracted many advocates and which is at present one of the most generally accepted theories concerning the nucleus of bacterial cells He wrote "I have assembled above a number of examples in which chromatic material, which is diffusely distributed throughout the cell, is present in addition to the cell nucleus, and which temporarily substitutes solely for the nuclear apparatus Herewith we have the possibility of organisms which have perhaps no true permanent nuclei but, in lieu of nuclei, chromatin bodies which are interspersed wholly or partly in the protoplasm Bacteria and *Oscillatoria* seem to me to be such organisms"

Schaudinn (162) studied a giant species, *Bacillus bütschlii*, from the intestinal contents of the kitchen roach, *Periplaneta orientalis* He saw no differentiation into central body and peripheral cytoplasm comparable to that described by Butschli for *Spirillum volutans* On the other hand, the cell contents consisted of alveolar cytoplasm in which there were scattered chromatin bodies during the greater part of the life history A violent fountain-like streaming of the granules which continued for several minutes occurred in cells immediately preceding spore formation As the streaming gradually subsided, the granules became arranged in the form of a wreath-like filament extending from pole to pole in the median axis of the cell A few of the granules in the polar position then united to form a rather large nucleus-like body which he regarded as a true nucleus and the beginning of the spore According to his interpretation, the cell nucleus consists of scattered chromidia during the vegetative phase of the life history, and a true nucleus is organized only at the time of spore formation

There are very good reasons to doubt his identification of the

stainable granules as chromatin bodies. Unfortunately, he made no microchemical tests to determine their nature, and since the organism could not be cultivated and has not been since by other investigators who have searched for it in the animal host, the precise nature of the bodies is not known. Concerning the behavior of the granules he said: "During its development easy distinction is made between the nucleus and cytoplasm. During the period of the inactive spores, this differentiation is lost (how, we do not know), for the young sporelings show no separation of nucleus and protoplasm." We now know that such behavior is typical for spore-forming species which deposit volutin, and there seems, therefore, little reason to doubt that the granules of *B. bütschlii* were grains of volutin. More recently, Lindegren (102) suggested that the granules might have been true nuclei produced by multiple division, but their behavior, origin *de novo* in the vegetative cells, does not support this view. The organization of a true nucleus at the time of spore formation may also be questioned. This will be discussed in a subsequent section. In the light of more recent investigations and in view of the fact that Schaudinn's theory of the nucleus has been widely accepted, the severe criticism by Meyer (118, 119) together with the reply by Schaudinn (163) is of great interest.

Guilliermond (55–57, 59–62) over a period of many years has been the most able advocate of the chromidial theory. He studied various species and, at times, seemed to give some support to the notion of a central body, but he always maintained that the bacteria do not possess a typical nucleus like that of higher plants. In 1917, he said: "The conclusion, to my mind, would be that while some bacteria may contain a rudimentary nucleus whose existence is nowhere else precisely demonstrated, so far, in the great majority of species, nothing more has been found than a diffuse nucleus consisting only of grains of chromatin scattered through the cytoplasm."

In his study of various spore-forming species, *Bacillus mycoides*, *Bacillus radicosus* and others, Guilliermond (55) observed that young cells present a homogeneous appearance and are uniformly stained with no great differentiation. Toward the eighth

hour of development, the cells show clearly their structure which is changed in appearance, the cytoplasm becomes vacuolated and displays a fine alveolar structure The web contains in its meshes small, highly stainable granules which seem to consist of chromatin To one who has become familiar with the structure of *B mycoides*, at different stages of the growth cycle, it is obvious that the description is accurate, but the identification of chromatin bodies is erroneous The change from the homogeneous structure of young cells to the vacuolated alveolar condition of older cells, in this species, is due to the deposition of fat bodies, while the so-called chromatic bodies are compressed cytoplasm Mencl (115) studied the cell structure of *Azotobacter chroococcum* He observed that the cell body shows a characteristic honey-comb appearance in which the lattice-like walls are dull, while the enclosed spaces are filled with a highly refractile mass This is a very good description of old, fat-filled cells of this species He saw also deeply stainable grains of volutin which he mistook for chromatin bodies In general, the evidence presented by other investigators (20, 138, 167) in support of a chromidial nucleus is not convincing

5 *Polymorphic nucleus* Amato (7) studied the structure of *Bacillus mycoides* in preparations stained *intra vitam* with brilliant cresyl blue He reported a single large nucleus in the spores and young rods, but in older cells the nucleus appeared to break up to form numerous chromidia He suggested that the variability in form might account for the various conflicting views concerning the nucleus

Dobell (35) wrote at length on the nucleus of various unknown bacteria which occur in the intestinal contents of frogs, lizards and other animals He concluded that the nucleus may occur as a single vesicular body, scattered chromidia, or chromatin filaments depending on different stages of development There is no evidence that he made suitable tests to distinguish between nuclear structures and cell inclusions The illustrations, reproduced in colors, suggest that many of the cells contained fat bodies as well as grains of volutin and that some of the cells were yeasts or other low fungi His observations, limited to stained

films from raw mixtures, afforded no adequate basis for his conclusions

Other papers which might be considered here are discussed in the section dealing with the diffuse nucleus

6 *A true nucleus* The theory that bacteria possess true nuclei has been supported by many of the most able investigators, but this does not mean that the several reports are mutually confirmatory There is still much doubt concerning the real nature of some of the so-called true nuclei which have been described In order to prove the existence of a true nucleus in bacteria, it must be shown that the organism belongs to the bacteria rather than yeasts or other low fungi, that a definite particulate body, differentiable from the cytoplasm, occurs constantly in each cell, that genetic continuity of the body occurs in nuclear and cell division, and that the body in question is not a cell inclusion, vacuole, spore primordium, a cytoplasmic body, or an artifact It seems needless to add that such rigid criteria have not been applied generally and that many invalid claims for the discovery of true nuclei have been made

The most generally accepted proof for the occurrence of a true nucleus in bacteria is found in the work of Vejdovsky (188) who studied *Bacillus gammari*, a species which he found in *Gammarus zschokkei* There seems to be no doubt that the body described by Vejdovsky is a true nucleus which possesses a nuclear membrane, chromatin bodies, and divides karyokinetically On the other hand, the identification of the organism as a bacterial species was seriously questioned by many (52, 123, 150, 162, 185) The consensus of best opinion appears to be that the organism is a yeast, *Cryptococcus gammari* Mencl (114) described comparable nuclei in bacteria which occur in the intestinal content of the cockroach, but his work was severely criticized (57, 123) and has not been generally accepted

Meyer (116, 117, 122, 123) studied the cell inclusions and the nucleus of *Aerobacillus polymyxa* (*Bacillus asterosporus*), *B tumescens*, and *Clostridium butyricum* (*B amylobacter* Meyer and Bredemann) In each of these species, he observed a small, spherical, colorless, refractile body, about 0 3μ in diameter,

which could be readily differentiated from fat, volutin, glycogen, and cytoplasm by differences in microchemical reactions and stainability According to his interpretation, the body should be called a true nucleus The nucleus occurred in the spore primordia, mature spores, and in the young rods produced at germination of spores In vegetative cells, there were usually two to six free nuclei, but he was not able to trace stages in nuclear and cell division Meyer's methods deserve brief mention The microchemical reactions for the determination of various cell inclusions have already been mentioned In his first studies, he stained the cells *intra vitam* with formol-fuchsin, but in the later study of *C butyricum* he employed various fixing agents and, after washing, stained wet mounts with iron alum hematoxylin He stated with emphasis that dried fixed films are not suitable for the demonstration of nuclei

Because of his great prestige, Meyer's conclusions were widely accepted but have not gone wholly unchallenged Guilliermond (59) commented as follows "It seems to be established, however, that the majority of the elements noted by Meyer are not nuclei but reserve products common among *Protista* and known as metachromatic corpuscles" This criticism could doubtless apply to the "nuclei" of *Aerobacillus polymyxa*, for volutin is deposited by this species It could not, however, apply to *B tumescens* which deposits fat bodies only or to *Clostridium butyricum* in which the cell inclusions are glycogen and iogen Neither of these could be confused with Meyer's nuclei The most damaging criticism was given by Zettnow (201) who studied *B tumescens* by Meyer's method and confirmed his observations However, Zettnow regarded the bodies in question as cytoplasmic structures rather than nuclei Complete confirmation of Meyer's work was reported by several of his students (39, 52, 131) Preisz (153) reported similar nuclei in *Bacillus anthracis*

Swellengrebel (180, 181) described zigzag and spiral filamentous nuclei in *Bacillus maximus buccalis*, *Spirillum giganteum* and other bacterial species, similar observations were recorded by Dobell (35), Dimitroff (32) and Paillot (138) Swellengrebel's work was however severely criticized (55, 123) Quite recently

Lewis (100) observed spiral arrangement of the stainable material in *Spirillum volutans* but was not able to confirm Swellengrebel's opinion that the filaments consist of chromatin The spirally arranged substance appears to be nothing but compressed cytoplasm lying between the numerous non-stainable fat bodies which are present in all species of *Spirillum*

Stoughton (177, 178) studied the structure and reproduction of a plant pathogen, *Phytomonas malvacearum*, which causes angular leaf spot of cotton Each cell contained a single, spherical, centrally located body which could be differentiated from cytoplasm by *intra vitam* staining Because of its constant occurrence, size, position in the cell, staining reactions, and division by constriction, he regarded the body as a true vesicular nucleus The nuclear nature of this body has been questioned by various investigators Dufrénoy (37) identified it as a vacuole, Petter (142) obtained diffuse staining of the rods by Feulgen's method, Guilliermond (62) regarded the body as a metachromatic corpuscle (volutin)

Hollande and Hollande (77) wrote at great length on the structure of various bacterial species including *Eberthella typhosa, Escherichia coli, Mycobacterium tuberculosis* and *Bacillus anthracis* They introduced the terms nucleosome, paranucleosome, and metanucleosome to designate structures which were differentiated from cytoplasm by means of a special staining method The nucleosome, a minute nucleus-like body, occurs in all cells, divides by constriction, and stains blue with the eosinate of methylene blue, the paranucleosome, an eosinophilic body, closely associated with the nucleosome and often obscuring it, divides into several small granules during cell division, the metanucleosome, an irregular basophilic body, surrounds the paranucleosome

Hollande (74) observed these organules in the cells of various species from the intestinal content of animals as well as in pure cultures of well known species He seems to have reached no very definite conclusions concerning the nature of the bodies but offered several possible interpretations The constant occurrence of the nucleosome in all cells and its characteristics are indicative of a true nucleus The transitory nature of paranucleosomes

and metanucleosomes suggests reserve substances elaborated by the cell protoplasm

Barnard (14) photographed cells of *Bacillus mycoides*, *B megatherium*, *Staphylococcus aureus* and *Serratia marcescens* by ultraviolet light and obtained images which, according to his interpretation, suggest that bacteria contain a nucleus which undergoes mitotic division Wyckoff and Ter Louw (195) employed similar technique in photographing cells of *Bacillus subtilis* and called attention to the absence of any structures which could be regarded as nuclei The theory of a true nucleus is supported by a number of observers (9, 18, 40, 130, 139, 165)

The theory of a true vesicular nucleus has received some support from investigators who employed Feulgen's reaction for differentiation da Cunha and Muniz (29) observed, as a rule, two stainable granules in young cells of *Bacillus anthracis* Stille (176) saw discrete stainable bodies in various species of spore-forming bacteria, *Azotobacter* and *Sarcina* The number and arrangement in the cell were not influenced by methods of cultivation, the bodies appeared to divide by constriction and for these reasons were regarded as true nuclei There is no indication that he distinguished between nuclei and inclusion bodies although we know that *Azotobacter chroococcum* deposits volutin

Piekarski (143–145) demonstrated "nucleoid" bodies in cells of *Escherichia coli*, *Salmonella paratyphi*, and *Serratia marcescens* by means of Feulgen's reaction and the electron microscope Cells from young cultures contained 2 nucleoids while other cells contained a single body The bodies appeared to divide preceding cell division and to consist of thymonucleic acid, since the ultraviolet absorption spectra of nucleoids and known nuclear substance were identical Piekarski and Ruska (146) studied the structure of several bacterial species by means of the electron microscope Electron micrographs of cocci, sarcinae, and spore-forming species show very little or no structural differentiation In cells of *Pseudomonas aeruginosa* and some other non-spore-forming bacteria, granular bodies similar to the nucleoids demonstrated by Feulgen's reaction are shown

7 *The diffuse nucleus* The theory that the bacterial nucleus

consists of finely divided particles of chromatin uniformly dispersed in the cytoplasm was proposed by Zettnow (201) although he had formerly spoken in favor of a central chromatin-containing structure, the central body The term diffuse nucleus is generally employed to denote a "nucleus" which is so finely divided and dispersed as to become undifferentiable from the cytoplasm Some writers have also spoken of scattered visible chromidia as a diffuse nucleus In order to avoid ambiguity, the term diffuse nucleus is used here to denote only the condition in which chromatin in the finely divided state is uniformly dispersed in the cytoplasm The term chromidial nucleus or chromidial system has already been applied to the condition in which the nucleus has been supposed to exist as scattered visible chromatin bodies

Several investigators have supported the theory of a diffuse nucleus Zettnow (201) based his conclusions on the fact that differentiated bodies invariably proved to be cell inclusions or cytoplasmic structures Thomas (182) described a new species, *Bacillus calmette,* in which the chromatin was uniformly dispersed or, at times, became separated from the cytoplasm to form visible units of pure chromatin or chromatin mixed with other bodies

The theory has received some support from investigators who employed Feulgen's reaction as a method of differentiation Feulgen and Rossenbeck (43) obtained negative results and concluded that bacteria do not contain thymonucleic acid Westbrook (192) was not able to obtain positive reactions with yeasts and bacteria Stapp and Bortels (172) saw diffuse staining of *Phytomonas tumefaciens* with no morphological differentiation Voit (190) obtained positive reactions with thick films Pietschmann and Rippel (148) reported uniform distribution of the stainable substance in normal cells of *B mycoides* Cultivation in media containing lithium chloride or magnesium sulphate resulted in abnormal cell forms in which the stainable substance became separated from the cytoplasm and appeared in the form of definite bodies

Imsenecki (78–80) maintained that a diffuse nucleus occurs in all bacteria and corresponds to a stage in the evolution of the nucleus at which chemical differentiation of nuclear substance has

already occurred, while the physicochemical conditions necessary for morphological differentiation of nuclear structures have not yet developed He believed that the dispersed chromatin can aggregate at certain stages in the life cycle to form visible units which may again break up into fine granules

Pokrowskaja (151) studied *Bacterium pestis* Cells growing as parasites showed a diffuse reaction, while under saprophytic conditions the stainable substance became aggregated into a definite nucleus-like body which was capable of amitotic division Milovidov (125) observed diffuse staining in young cells of *Bacillus mycoides*, *B megatherium* and *B anthracoides*, but in older cells the substance united to form compact bodies

The service which Feulgen's reaction has rendered in the study of bacterial structure is difficult to evaluate, since nucleic acid is frequently present as a reserve material and the reaction is not sufficiently intense to give a clear differentiation of minute structures. Margolena (108), Knaysi (86) and others have questioned the specificity of the reaction The investigations seem to prove that many bacteria contain thymonucleic acid which is generally in the form of minute granules but may, under some conditions, separate out and become aggregated into definite bodies which resemble true nuclei It could be argued that the drastic treatment required in this method causes plasmolysis or other artificial alterations in the cell structure This has been discussed at length by Stille (176) Schaede (161) who stained bacteria and actinomycetes by Feulgen's method thinks that the stainable substance, diffuse or in the form of granules, is not true chromatin or gene material and that it probably functions as reserve food

8 *The chromosome theory* Lindegren (102) has formulated a theory of the bacterial nucleus based on our knowledge of the cytology and genetics of higher organisms the gene is the fundamental particulate living unit and without it life is impossible, a linear aggregate of genes is the chromosome and an aggregation of chromosomes is the nucleus, the chromatin itself is not the hereditary substance but consists of inert material in which the genes are embedded, the genes maintain a fixed position in regard to each other, synchronize in division, and are distributed in such a

manner that each daughter cell receives its full complement of genes Accordingly, a nucleus reduced to its lowest essentials might consist of a single gene string encrusted with chromatin, or a single naked gene string A nucleus of this type could occur in the bacteria, but a diffuse nucleus is not possible since the genes do not diffuse but maintain an orderly position in the gene string

It seems probable that such a simple nucleus would take the form of a small granule or a rod-like body rather than a definite vesicle with a membrane separating it from the cytoplasm Lindegren has shown by diagrams drawn to scale, that a space 0 2μ in diameter is sufficient to accommodate a gene string of maximum theoretical size, and provide ample room for an orderly transmission of genes to the daughter cells

Some evidence favorable to this chromosome theory is found in the most recent investigations of the subject Lindegren and Mellon (101) described a diplococcus in which the nucleus consisted of a single haploid chromosome which contained seven chromomeres

According to Badian (13) the nucleus of *Bacillus subtilis* consists of a single rod-like, haploid chromosome which divides lengthwise preceding cell division The daughter chromosomes move apart before the transverse membrane is formed and each of the new cells receives a single chromosome In spore formation, the chromosome divides in the usual manner, the two segments arrange themselves end to end in the median axis of the cell, and fuse end to end to form a single bivalent chromosome This is followed by two successive, longitudinal divisions resulting in four haploid chromosomes, one of which becomes enclosed in the young spore while the three remaining in the cytoplasm are eventually lost Allen, Appleby and Wolfe (4) described a somewhat similar series of events in the division and spore formation of an unidentified species of *Bacillus* isolated from grass Each vegetative cell contains a single haploid chromosome, but the spores may be either haploid or diploid depending on the manner of origin Transition from diploid to haploid condition occurs in spore germination

Summary of the theories Much of the conflicting evidence

having been assembled, we are now faced with the obvious difficulty of reaching a satisfactory conclusion There is probably no one fully competent to perform this difficult task The writer believes that the claims for a naked nucleus, a central body, a chromidial system, a polymorphic nucleus, and a true vesicular nucleus are based on faulty evidence and must be rejected Much of the confusion has been caused by failure to distinguish between volutin and chromatin Due to their nucleic acid content these substances react to the usual nuclear stains in much the same manner Suitable tests for volutin have long been known, but there has been a marked tendency among investigators to regard all deeply staining bodies as nuclei or chromidia Unless specific tests for volutin have been made, the occurrence of stainable granules in the cells of bacteria is not significant This criticism alone is sufficient to disprove the claims concerning the nucleus of such species as *Spirillum volutans*, *Azotobacter chroococcum Bacillus bütschlii* and many others

Although the occurrence of volutin has been the principal cause of error, there has been much confusion concerning the nature of the stainable substance in fat-depositing species When cells containing fat bodies are fixed and stained by the usual methods, the compressed protoplasm appears as deeply stained compact masses, and zigzag or spiral threads which in many cases have been described as nuclei The picture is even more complicated in species which deposit both fat and volutin It is impossible to escape the conviction that many observers, failing to recognize the true nature of the stainable structures, have projected a subjective element into the problem

The more recent theory that the nucleus consists of minute particles of chromatin uniformly dispersed in the cytoplasm is based principally on results obtained by Feulgen's method of staining No very definite conclusions can be drawn from the various conflicting reports which have appeared

If the bacterial cell contains no demonstrable nucleus, the possibility still remains that the nucleus consists of an invisible structure essentially the same as the gene strings in the chromosomes of higher organisms, but devoid of the usual encrustation of chroma-

tin In the present state of our knowledge no final conclusions concerning the nature of the nucleus can be drawn It appears, however, that we may now discard much of the uncritical work of the past and begin to think in terms derived from the more certain knowledge of the genes of higher organisms Whether the protoplasm of the bacterial cell is undifferentiated, as claimed by Fischer and others, or consists of invisible genes and cytoplasm has not been definitely determined In the light of all the experience of the past it seems highly probable that any claims based on cytological methods will not be found wholly convincing Whether our knowledge of the hereditary mechanism of bacteria can be enhanced by genetical technique is still a question

III REPRODUCTIVE STRUCTURES

1 *Gonidia* The theory that bacteria reproduce by means of small coccus-like bodies, gonidia, borne within cells of normal shape or in large specialized cells, gonidiangia, has had many advocates The theory, now regarded as an established fact by many bacteriologists, is based in part on the observation that bacterial cells frequently contain granular bodies which appear to escape from the mother cell and develop into cells of the parental form Indirect evidence for the occurrence of minute reproductive bodies, smaller than ordinary vegetative cells, has been obtained by filtration experiments Theoretically, there are some reasons to believe that such a method of bacterial reproduction is possible We have long known that *Sphaerotilus dichotomus* reproduces by forming motile cells which escape and develop into the thread-like form Similarly, reproduction by means of zoospores, reproductive cells formed in sporangia by free cell formation, is of common occurrence in many fungi and algae Whether any of the true bacteria reproduce by the formation of internal cells, analogous to those produced by algae and fungi, is an open question although there is an ever increasing volume of evidence which supports the theory It is true, however, that much of the evidence is not very convincing and that some of it has been discredited

The opponents of the theory have maintained that the so-

called gonidia are not capable of germination, that they are, in many instances, cell inclusion bodies or compressed cytoplasm, and that growth in filtrates is not conclusive evidence of a gonidial method of reproduction The literature of the subject has become rather extensive, and there is not sufficient space here for an adequate critical review We shall be obliged, therefore, to select a few of the most thoroughly studied species and present the evidence for and against this theory of reproduction

Jones (82) reported deeply stainable, non-filtrable, reproductive granules in the cells of *Azotobacter sp* Lohnis and Smith (105, 106) described stainable and non-stainable, filtrable reproductive granules Lewis (98) identified the granular bodies as grains of volutin and fat bodies Jones (82), Roberg (157) and Lewis (98) obtained negative results by filtration methods It appears that the advocates of gonidial reproduction in *Azotobacter* confused cell inclusion-bodies with gonidia and reached unwarranted conclusions

Few genera of bacteria have been so thoroughly studied as *Rhizobium*, and there is probably no genus in which reproduction by gonidia has been so generally accepted Support is found in the early work of Morck (127), and in later publications by Bewley and Hutchinson (19), Gibson (48), and others It would appear that here, if any place, the evidence is so overwhelming as to compel acceptance The case of gonidial reproduction was well stated by Thornton (183) who wrote as follows "Thus, in *B radicicola* the rod-shaped cells at first stain evenly, but later the stainable material becomes segregated into bands These develop into spherical granules of which a single cell may contain from 1 to 6 Rupture of the mother cell releases the granules which at first are usually non-motile, but later swell in size and become actively motile All stages in the elongation of the cocci to form the evenly staining rods can be found " The writer (99) attacked this theory on the ground that the banded condition is not caused by free cell formation but by the deposition of non-stainable fat bodies which restrict and compress the cytoplasm to form the stainable bands The small cocci and ovoid cells seen in old cultures are small vegetative cells caused by fission during the period

of declining growth There seems to be no reason to recede from this position

Reproduction of *Bacillus mycoides* by means of filtrable gonidia was described by Nyberg (135), and Oesterle and Stahl (137), but this conclusion was opposed by Stapp and Zycha (173), Lewis (96) and den Dooren de Jong (30) The stainable bands and bars in this species, as in rhizobia, are conditioned by fat bodies Haag (64) reported the occurrence of gonidia in *Bacillus anthracis* Rettger and Gillespie (155) and Knaysi (85) found no evidence to support the theory of filtrable gonidia in other sporogenous species including *B megatherium*, *B vulgatus* and *B mesentericus* The evidence is, therefore, mostly against gonidial reproduction in this group of well known species Quite recently Allen, Appleby, and Wolf (4) reported filtrable gonidia in an unidentified species of *Bacillus* isolated from grass

Concerning gonidial reproduction in mycobacteria, there have been so many publications that no attempt can be made to review them here Many workers have regarded Much's granules as viable, filtrable units while others have identified them as products of cell degeneration If it is true that the highly refractile granules, first described as endospores by Koch (90) are fat bodies, as claimed by Grimme (52), Meyer (123), and Hartman (66), then it would seem to follow that the stainable bands are nothing but compressed cytoplasm as in rhizobia and other fat depositing species A still further study of this group is needed to establish the identity of the refractile bodies and the stainable elements

The evidence in regard to gonidial reproduction in *Corynebacterium* is somewhat contradictory Mellon (111) studied a species originally isolated from a case of Hodgkin's disease and described small motile bodies which became free and gave rise to new individual cells Bergstrand (17) studied the same species but failed to confirm the occurrence of such reproductive bodies More recently, Groh (54) reported that the granules which stain by Neisser's method are living units which burst the maternal rod and develop into new cells This appears to contradict all that is known concerning the nature of Neisser's granules

Löhnis and Smith (105), Enderlein (40) and Hadley, Delves and Klimek (65) maintained that gonidial reproduction occurs in all species of bacteria, but the evidence for this claim can not be regarded as very substantial. Whether gonidia occur in all, some, or none of the species of true bacteria is a difficult question to answer on the basis of our present knowledge. The writer believes that the positive results obtained by filtration experiments have little or no value and that the answer must be sought in the study of cell structure. Until we have seen a vegetative cell break up into granules, liberation of the granules from the mother cell, and development into new vegetative cells, this method of reproduction must be regarded as a theory rather than an established fact. There seems to be no doubt that many investigators have confused other structures with gonidia.

2. *Gametes*. The hypothesis that bacteria reproduce by sexual methods has received some support, but the evidence is not extensive and has not been very generally accepted. Among the early workers Schaudinn (162) observed an abortive division of the rods and violent streaming of granules preceding spore formation in the disporic species, *Bacillus butschlii*. According to his interpretation, spore formation in this species is preceded by autogamic conjugation. Dobell (33) supported this conclusion but in a later study of the problem (34) reversed his former opinion, and still later (35), he rejected all theories of sexual reproduction in bacteria. Stewart (175) believed that asexual reproduction eventually comes to a close in a colony of bacteria and is followed by "an outburst of conjugation." He described the process as autogamous. The chromosome behavior observed by Badian (13) and Allen, Appleby and Wolf (4) supports the theory of autogamic conjugation.

Various investigators have described isogamic conjugation. Pothoff (152) described tube-like processes connecting the conjugating cells of *Chromatium okenii*. The cells of this species are very large, and if the process occurs the phenomena could probably be followed. His photographs are not convincing, and the conclusions were attacked by Krasil'nikov (91) who saw nothing but incompleted cell divisions. The same applied to the conjuga-

tion of *Azotobacter* described by Löhnis and Smith (105) Mellon (112) presented evidence to show that isogamic conjugation, similar to that of yeasts, occurs in *Escherichia coli* More recently, Hollande and Hollande (77) described a somewhat similar fusion of isogametes in *Mycobacterium tuberculosis* Nyberg (135) saw very tiny motile isogametes liberated from cells of *Bacillus mycoides* Stoughton (178) reported isogamic conjugation in *Phytomonas malvacearum*

Although most advocates of the theory of sexual reproduction in bacteria have supported the more primitive methods, autogamic and isogamic conjugation, Enderlein (40) claims to have observed fusion of heterogametes, sperm cells (spermits) and egg cells (oits) in *Vibrio cholerae* According to his account, the gametes originate from gonidia by a process of reduction analogous to that of higher organisms Almquist (6) reported somewhat similar reduction division in *Eberthella typhosa* Sexual reproduction by conjunction, multiple fusion of cells, was reported by Löhnis and Smith (105) and Appleby (9)

If the bacteria reproduce by sexual methods, it should be possible to cross closely related strains or species and determine something concerning the genetical behavior There is not, however, much evidence to support the theory that hybrids occur in bacteria Almquist (6) reported success in crossing *Shigella dysenteriae* and *Eberthella typhosa* Nyberg (136) believed that *B mycoides* is a hybrid and that dissociation in this species is due to Mendelian segregation Stewart (175) reported autogamic conjugation and segregation in *B coli-mutabile* Mellon (112) believes that rearrangement of chromatin by a sexual process is a chief cause of bacterial variation Sherman and Wing (168) attempted to cross strains of *Escherichia* but abandoned the experiment with inconclusive results

There appears to be no conclusive evidence that sexual reproduction occurs in bacteria It may be noted also that botanists have not been able to prove sexual reproduction in the *Cyanophyceae*, although these plants are much larger than bacteria and more favorable for cytological study

3 *Endospores* Several conflicting methods of endospore for-

mation have been described by different investigators According to Koch (88) spores result from the upgrowth of a tiny, refractile, non-stainable granule Although some support for this theory is found in the literature, it is now generally regarded as erroneous

Following the work of Ernst (41, 42) and Babes (10), the theory that spores are formed by the fusion of numerous sporogenic granules attracted many advocates and is still held by some bacteriologists Support is found in the work of Bunge (23), Dobell (33–35), Schaudinn (162) and Nyberg (135) Much of the older as well as the more recent work is opposed to this theory We now know that the so-called sporogenic granules of earlier writers are volutin grains, fat bodies, or glycogen and that they occur in many species which do not form spores Moreover, there are many species in the genus *Bacillus* which do not contain granules and could not, therefore, form spores by this method There is no sound cytological evidence that endospores are formed by the fusion of sporogenic granules

A method of spore formation which seems to have been proved beyond reasonable doubt was described by Peters (140) and confirmed by Meyer (116, 117), and others (15, 52, 97, 153, 195) The spore is formed from a clear, hyaline, polar spore primordium which is set off from the remainder of cell by a membrane The spore results from a condensation of the substance contained in the spore primordium The granules, if present, are not concerned directly in spore formation, although they may furnish nutritive material which is digested and absorbed by the developing spore During the early stages before the spore body has become fully condensed, it stains readily with aniline dyes and for this reason resembles a nucleus There seems little reason to believe that the formed nucleus of *Bacillus bütschlii*, reported by Schaudinn (162) was anything but an immature spore

IV CELL DIVISION

Various methods of bacterial cell division have been described Knaysi (86) classified the methods under three headings (1) plate formation, centrifugal or centripetal, (2) cytoplasmic retraction and (3) constriction Support is found in the litera-

ture for each of these methods In the most recent study of the subject, Knaysi (87) found no indications of division by constriction or the formation of a cell plate with subsequent splitting His observations appear to prove that the cell divides by cytoplasmic retraction and subsequent formation of two transverse cell walls which are separate from the beginning A rather full review of the previous literature is given

V THE CELL MEMBRANE

There has been much confusion concerning the nature of the outer envelope of the bacterial cell and its relation to the protoplasm The term membrane as generally employed has no very definite meaning, and the term ectoplasm or ectoplast is even less satisfactory Since the early writings of Zettnow, some bacteriologists have employed the term ectoplasm to designate the outer portion of the cell and endoplasm to denote the cell contents The following quotation from Rideal (156) is typical of the many brief descriptions which occur in recent textbooks "In general, bacterial cells may be regarded as a chromatin network in an emulsoid protoplasm, the endoplasm, the whole being enclosed in a semi-permeable membrane, the ectoplasm " The term ectoplasm has also been used by Preisz (153), Eisenberg (38) and Gutstein (63) to denote the whole outer envelope Churchman (26) introduced the terms cortex and medulla which have caused additional confusion

The writer agrees with Knaysi (86) who suggested that we abandon this confusing terminology in favor of terms which are universally employed in dealing with other plant cells It could be argued that the structure of the bacterial cell is not analogous to that of other vegetable organisms and that a different terminology is necessary The evidence must therefore be considered Knaysi (84) stained *Bacillus subtilis intra vitam* with dilute aqueous solution of crystal violet He observed a purple outer cell wall surrounding a dark violet membrane which encloses a deeply staining cytoplasm but of much lighter shade The cell wall could be seen much more clearly in cells plasmolyzed by mounting them in 25 per cent sodium chloride solution prior to staining

According to his observations the cell wall has only a slight affinity for dyes, it stains a clear blue with methylene blue but has no affinity for iodine The cytoplasmic membrane, on the other hand, is hyperchromatic and takes up dyes with great avidity It is colored dark brown with iodine More recently, Knaysi (87) has described a differential method of staining by which the cytoplasm, cell wall, and cytoplasmic membrane can be distinguished in fixed films

These experiments, together with the earlier work of Fischer (45), Grimme (52), Ellis (39), Swellengrebel (181), Meyer (123), and others proved that the protoplast of the bacterial cell, like other plant cells, is not attached to the cell wall but lies free within it In the fully turgid cell, the cytoplasmic membrane is in close contact with the outer wall and is not readily distinguished When the cells are suspended in a hypertonic solution, the cytoplasmic membrane is drawn in with the contracted cytoplasm while the more rigid cell wall retains its original form

Much has been written on the permeability of the bacterial membrane especially in its relation to dyes We know that the cellulose wall of higher plant cells is very permeable while the cytoplasmic membrane is semipermeable It is known that the protoplast of the bacterial cell functions as an osmotic system, precisely like that of other plant cells, and that the cytoplasmic membrane is semi-permeable According to Fischer (45), such a degree of division of labor as occurs in the higher plants has not been reached in the bacteria where communication between the organism and the outer world is regulated by two layers of medium permeability To what extent, if any, the true cell wall of bacteria functions as a selective membrane is difficult if not impossible to determine

The chemical composition of the cytoplasmic membrane as well as that of the cell wall is not very well known Knaysi (86) believes that the cytoplasmic membrane is made up chiefly of surface active materials, lipoids and lipoproteins, which accumulate to form a rather firm surface structure which may consist of several layers The cell wall is a firm, rigid, somewhat elastic structure as was shown by Ellis (39) for *Spirillum giganteum* and by the

ingenious needle dissection experiments performed by Wamoscher (191)

Claims have been made that the wall substance of some bacteria consists of cellulose, but there is little or no convincing evidence that such claims are valid The earlier literature was reviewed by Meyer (123) who advanced the theory that the substance is a hemi-cellulose The subject has been discussed more recently by van Wisselingh (187) The occurrence of chitin in the cell walls of various bacteria was denied by van Wisselingh (186) but was supported by Iwanoff (81), and Viehoever (189) van Wisselingh (187) found that chitin is a common component of the cell walls of molds but is not present in bacteria

The mucoid substances deposited as a clear zone external to the cell wall and variously designated as the slime layer, sheath or capsule have been studied more extensively than the wall itself The precise origin of the capsular material has long been a more or less controversial matter The earlier investigators generally regarded the substance as a product formed by swelling and gelatinization of the so-called ectoplasm Meyer (123) and Zettnow (201) attacked this theory and held that the substance is a secreted product In the light of more recent studies on the chemical nature of the gums produced by various species, this view is much more likely The chief interest manifested in capsules by cytologists has been in the matter of successful methods of staining There is little doubt that low affinity for dyes is due to the carbohydrate nature and that the capsular substance may adsorb stainable materials from blood serum or even from milk The extensive literature dealing with the chemistry of the capsular material and its relation to virulence and to immunological reactions, though matters of great importance, does not concern us here

Of considerable interest to the student of bacterial cytology are the investigations of Churchman (26) who maintained that the gram reaction is due to a protein-like, gram-positive substance, the cortex, deposited as a sheath around the inner gram-negative portion, the medulla Eisenberg (38) and Gutstein (63) described a similar surface layer differentiable from the remainder of the cell

body by various staining methods Churchman's conclusions were attacked by Burke (24) who could find no evidence that gram-positive bacteria possess a cortex which stains differentially by Gram's method Stearn and Stearn (174) expressed some doubt concerning the presence of an external cortex Knaysi (84) regarded the cytoplasmic membrane as the structure which corresponds to Churchman's cortex and is responsible for the gram reaction

Although our knowledge of the structure of the bacterial cell may not be perfect, enough is known to warrant a more uniform usage of the terms employed in the general domain of plant cytology Accordingly, the bacterial cell consists of a protoplast encased in a non-protoplasmic cell wall composed of ergastic substances of unknown identity The surface of the protoplast is a differentiated semi-permeable cytoplasmic membrane which encloses the cytoplasm Within the cytoplasm there are sap vacuoles and in some cases various granular inclusion bodies The nucleus of the protoplast probably consists of a structure analogous to a chromosome rather than to a true vesicular nucleus

VI FLAGELLA

The earlier investigations concerning flagella were directed principally at the problem of staining Robert Koch (1877) discovered the first successful method by which flagella could be made visible by staining He employed "Extractum campech," a crude extract of logwood (*Lignum campechianum*) The staining action was enhanced and made permanent by treating the stained films with dilute chromic acid

Loeffler (103) discovered a better method based on the use of a mordant consisting of a mixture of tannic acid, ferrous sulphate, and basic fuchsin He stained the mordanted cells with carbol fuchsin Zettnow (200) improved the silver impregnation method which had been previously employed and devised a valuable method especially suitable for photographic purposes

Modifications of Loeffler's method have been made by Shunk (169), Gray (51), Leifson (95), Maneval (107) and many others The most complete study of mordants is probably that of Maneval

who prepared 24 different solutions which gave excellent results with various species Successful mordants contain much material in a colloidal state, and it appears probable that the principal factor involved in staining is adsorption Maneval suggested that the size and electrical charge of the colloidal particles as well as the H-ion concentration are important factors It is of interest to note that Loeffler recommended the use of caustic soda or dilute sulphuric acid as a "corrective solution" for staining some species

The several factors causing variable results with flagella stains have been discussed in considerable detail by Wright (194) A factor which appears not to have been fully appreciated concerns the nature of the glass surface Erwin F Smith (170) called attention to the fact that thoroughly cleaned cover glasses sometimes give trouble because the surface of the glass itself is at fault More recently, Conn and Wolfe (27) stressed the importance of flaming the slides until an orange color appears in the flame The surface appears to be improved by this method Shunk (169) recommended chemical rather than heat fixation of films

The discovery of successful staining methods stimulated investigation of other problems concerning flagella Much interest has been manifested in the manner of origin from the cell body Migula (124), Zettnow (199, 201), Schaudinn (162), Marrassini (109) and others believed that flagella originate from the membrane, while Fischer (45), Ellis (39) and Meyer (123) held that they originate in the cytoplasm and grow out through openings in the cell wall This latter view was modified somewhat by Reichert (154), Fuhrmann (46), and Yamamoto (196) who described the origin from an internal granule comparable to the blepharoplast of other flagellated cells More recently, Leifson (95) supported cytoplasmic origin, and Enderlein (40) reported the occurrence of a blepharoplast (centriolit) in the sperm cells (spermits) but not in ordinary vegetative cells It is probably impossible to obtain any very definite cytological evidence as to the precise manner of origin, but in the light of our present knowl-

edge of the cell structure, there is little or no support for the theory that flagella originate from the cell membrane The occurrence of blepharoplasts appears to be doubtful

The application of dark field illumination to the study of bacteria has enhanced our knowledge of the mechanism of flagellar motion This subject was thoroughly studied by Reichert (154) and more recently by Neumann (134), who stressed the importance of a suitable viscous mounting fluid According to Reichert (154), the force which propels the bacterial body is not due to lashing of the flagella as was supposed by Fischer (45) but to a rhythmic contraction which moves helicoidally over the surface, the action is comparable to that of a screw rather than an oar Pijper (149) has shown that the movements to right or left are controlled by changes in the angle which the flagella make with the cell body, they act as a rudder as well as a propeller

Recent studies by dark field methods have caused some doubt concerning the occurrence of peritrichous flagellation According to the observations of Pijper (149) *Eberthella typhi*, *Proteus vulgaris* and similar species swim by means of a long "tail," formed by the twisting together of two rather broadly coiled flagella which are attached to the cell near its middle He regards as artifacts the usual appearances of peritrichous flagellation seen in stained fixed films Pietschmann (147) observed subpolar flagella on living cells of *Bacillus subtilis*, *B ellenbachensis*, *B ruminatus*, *E coli*, and *Serratia marcescens* He regards peritrichous flagellation as an illusion which is due to the appearance of subpolar flagella on chains of cells

Although we are not concerned here with the literature dealing with flagellar and somatic antigens, the dark field observations of agglutination made by Pijper (149) may be noted In the presence of flagellar antigen, the flagella become encrusted with a substance which causes them to adhere rather loosely when fortuitous entanglements occur This results in the formation of clumps which are readily broken down by shaking There appears to be no effect on the cell body On the other hand, the somatic antigen has no such effect on the flagella but acts on the

cell bodies causing them to unite in compact masses which are not readily separated by shaking In this connection the experiments of Craigie (28) on *Eberthella typhosa* are of special interest

Investigations by Piekarski and Ruska (146) and by Mudd, Polevitsky and Anderson (128) indicate that the electron microscope is valuable for the study of flagella Evidence obtained by Polevitsky suggests that the flagella of *Eberthella typhosa* and coliform bacteria may be tubular structures

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ANTAGONISTIC RELATIONS OF MICROÖRGANISMS[1]

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CONTENTS

Within recent years, there have appeared a number of reviews dealing with the antagonistic interrelations among various microörganisms, some of these have dealt with certain specific groups, and others with a great variety of organisms. It is sufficient to mention Holman (155), Buchanan and Fulmer (41), and Nakhimovskaia (231) on bacterial associations, Seitz (303) on mixed infections, Brown (40), Novogrudsky (240), Weindling (355), Porter and Carter (261) and D'Aeth (64) on competition among fungi, Garrett (118) and Garrard and Lochhead (114) on the interrelations between soil-borne and disease-producing fungi, Nakhimovskaia (230) on antagonisms between actinomycetes and bacteria, and Waksman (345) on associations and antagonisms among microörganisms in different habitats. In this review, an attempt is made to present the broad antagonistic relations between microörganisms living in association, either in simple mixed cultures or in complex natural populations, the

[1] Journal Series Paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Soil Chemistry and Microbiology.

significance of these associations in natural processes, their relation to disease production in man as well as in domesticated plants and animals, the chemical nature of the active substances produced, and finally, the nature of the antagonistic action

SURVIVAL OF HUMAN AND ANIMAL PATHOGENS IN SOIL AND IN WATER BASINS

Microbes capable of causing disease find their way into the soil and into water basins in very large numbers, either in the excreta of the infected host or in the dead and infected residues of the latter If one considers the millions of years that animals and plants have existed on this planet, one can only surmise the great numbers of microbes causing the numerous diseases of all forms of life that must have thus been introduced into soils and surface waters What has become of all the bacteria causing typhoid fever, dysentery, cholera, diphtheria, pneumonia, bubonic plague, tuberculosis and leprosy in man, mastitis and abortion in cattle, and of numerous diseases of other animals? This question was first raised by medical bacteriologists in the eighties of the last century The soil was searched for the presence of bacterial agents causing infectious diseases and responsible for epidemics The results obtained established beyond doubt that, with very few exceptions, organisms pathogenic to man and to animals do not remain alive in the soil for very long

It is true that a few disease-producing microorganisms are able to survive in soils for considerable periods of time One need only mention the organisms causing tetanus, gas gangrene, skin infections, actinomycosis and blackleg in cattle, coccidiosis of poultry, hookworm infections, trichinosis, enteric disorders in man To these must be added certain bacteria, actinomycetes and fungi, which cause a variety of plant diseases such as potato scab, root rots, take-all of cereal crops, and the damping-off diseases of vegetables However, the great majority of disease-producing microorganisms are able to remain in an active and reproducible state only for short periods outside of their respective hosts, and especially in soils and in natural waters It is sufficient to cite the fact that typhoid and dysentery bacteria, which are known to contaminate watersheds and water supplies, sooner or

later disappear No one now raises the question concerning the role of the soil as the carrier of these disease-producing agents or as the cause of severe or of even minor epidemics This rapid disappearance of disease-producing bacteria may be due to several factors, such as (*a*) unfavorable environment, (*b*) lack of sufficient or proper food supply, (*c*) destruction by predacious agents, such as protozoa and other animals, and (*d*) destruction by various saprophytic bacteria and fungi considered as antagonists

Frankland (108) was the first to establish that the typhoid bacterium may survive in sterilized polluted water or in pure deep well water for 20 to 51 days, but that it dies out rapidly in 9 to 13 days in unsterile surface water Jordan and his associates (171) found that *Eberthella typhosa* survived in sterilized tap water for 15 to 25 days, as against 4 to 7 days in fresh water, it died off even more rapidly (in 1 to 4 days) in raw river or canal water The degree of survival of this organism in water was found to be in inverse ratio to the degree of contamination of the water, the saprophytic bacteria being directly responsible for the destruction of the pathogen (292) Freshly isolated organisms survived a shorter time than laboratory cultures, and higher temperatures were more destructive than lower ones (163)

The presence of certain bacteria in water is often found to hinder the survival of *E typhosa* (336) When *Pseudomonas aeruginosa*, on the other hand, is present in drinking water, it may not be accompanied by any other bacteria (280) Media inoculated with this organism and with *Escherichia coli* gave, after 13 days cultivation, only cultures of the former, however, the two organisms can coexist in sterilized water *Vibrio cholerae* does not survive very long in fresh water, although long enough to cause occasional epidemics (139)

Typhoid and paratyphoid bacteria were found (368) to have only a very short life in sewage sludge, a reduction of 99% being reported after 6 hours' treatment of activated sludge (319, 153) There is a marked difference in the survival of different strains, Ruchhoft (290) having shown that whereas one strain died off very rapidly, two others died only in 8 to 10 days, and one survived for 13 days

The addition of typhoid bacteria to a well-moistened and

cultivated soil brings about their rapid destruction (207). The same phenomenon occurs when a culture of these organisms is added to that of a soil microbe. An antagonistic relation is often found to exist in some soils but not in others, this being traced to the presence of specific bacteria. Frost (110) also reports a marked destruction of typhoid bacteria added to the soil, 98 per cent of the cells being killed in six days; it is suggested that in the course of a few more days all these cells would have disappeared from the soil. This is also true of sea water (167). On the other hand, under conditions less favorable to the antagonists, the typhoid organism survives not only for many days, but even for months.

E. coli is rapidly crowded out by other organisms in manure piles (227) and in soil (298, 308, 349). The dysentery and typhoid organisms disappear rapidly in sea water, namely in 12 and 16 hours; the paratyphoid organisms have been found to survive for 21 and 23 days (330). Sea water appears to contain an agent, other than its salts, which exerts a bactericidal effect (372).

Under conditions prevailing in southern England, *Mycobacterium tuberculosis* was found (364) to remain alive and virulent in cow's feces, exposed on pasture land, for at least five months during winter, two months during spring and four months during autumn; in summer, no living organisms were demonstrated even after two months; under protection from direct sunlight, the survival period was longer. Bovine tubercle bacteria have been detected in soil and manure, and on grass up to 178 days after infection, but not later (204). When *M. tuberculosis* was added to non-sterile soil, it was slowly destroyed (278, 313); the plate count was reduced to about one-sixth of the original in one month (33). *Brucella melitensis* survived in sterilized tap water for 42 days, as compared to 7 days in unsterilized water; it survived in sterilized soil 72 days, as compared to 20 days in unsterilized soil (161).

In spite of the gradual and even rapid destruction of some pathogenic microörganisms in the soil, the survival of others presents important problems to farmers raising hogs, cattle, poultry and other domestic animals. In order to overcome this

condition, the rotation of crops is usually practiced, several years are usually required to render infected pastures safe for use A better understanding of the antagonists that are responsible for the rapid destruction of pathogenic organisms in the soil may throw light upon this problem and improve the methods of control

SYMBIOSIS AND ANTIBIOSIS

In a natural milieu, such as soil and water, which is inhabited by a mixed microbiological population, numerous relations of association and antagonism occur All organisms inhabiting such a medium are affected, directly or indirectly, by one or more of the other constituent members of this population These relationships were at first visualized as due primarily to competition for nutrients, as was well expressed by Pfeffer (256), who said "the entire world and all the friendly and antagonistic relationships of different organisms are primarily regulated by the necessity of obtaining food " De Bary, in 1879, was the first to emphasize the significance of the antagonistic relations among microörganisms (66) When two organisms are grown on the same substrate, one overcomes the other sooner or later, and even kills it The limited food supply in the culture medium was believed by many to be responsible for this (194), the fast-growing organism being favored as compared to the slow grower Kruse (187) also suggested that this is a problem of food competition When two organisms are capable of utilizing the same nutrients, but are differently affected by environmental conditions, such as reaction, air supply and temperature, the one that finds conditions more suitable for its development, will grow more rapidly and in time be able to suppress the other

However, it soon became clear that antagonism among microorganisms embraces phenomena other than mere competition for or exhaustion of nutrients E F Smith (309) pointed out, for example, that when two or more organisms live in close proximity, they may exert mutually antagonistic, indifferent or favorable effects According to Porter (260), the effects produced upon each other by fungi in mixed cultures may be due to the formation

of substances which exert detrimental or beneficial effects Lasseur (189) regarded antagonism as a very complex phenomenon a result of numerous and often little known activities, it influences the morphology of the organism, the capacity for pigment production, and various physiological processes

The terms "association" and "symbiosis" are used to designate mutually beneficial relations, as contrasted to "antagonism" and "antibiosis," which refer to a reduction in growth and in activities, as a result of the living of organisms in mixture (235) Bacteria may respond to such effects by exhibiting temporary or permanent modifications in their physiological characteristics (311) The morphology of diphtheria organisms may be influenced thereby, often accompanied by a reduction in virulence (151) Certain bacteria will form abnormal morphological cells under the influence of antagonistic actinomycetes (230), these changes are not hereditarily stable Pyocyanase brings about morphological changes in *B anthracis* (95) Penicillin, by inhibiting fission of bacteria, leads to abnormal growth of the cells, followed by autolysis (113) Different fungi will favor perithecia formation by other fungi and will influence the germination of ascospores (9, 276) Pigment formation by *P aeruginosa* may be weakened in the presence of other organisms, *E coli* may lose the property of fermenting sugar when grown together with paratyphoid bacteria (168) This type of antagonism has often been referred to as "functional antagonism" (231) The production of inactive lactic acid by *d*- and *l*-acid-producing bacteria, in the presence of certain anaerobes (323), as well as the formation of lactic acid by butyric acid bacteria, in the presence of other organisms (315), are other illustrations "Synergism" is used to designate the living together of two organisms, resulting in a change which neither alone could bring about (155)

The injurious effects of one organism upon another range from antagonisms, of varying degrees of intensity, to the living or preying of one upon the other, the latter phenomenon may be classified with pathogenicity and disease production Various types of antagonism have thus been recognized (231) 1 Antagonism *in vivo vs* antagonism *in vitro*, the former being often desig-

nated as antibiosis (189) 2 Repressive, bactericidal and lytic forms of antagonism, as well as antagonism of function *vs* antagonism of growth 3 Direct, indirect and true antagonism 4 One-sided and two-sided antagonism, antagonism between strains of the same species and among different species, or iso- and hetero-antagonism (88)

Duclaux (79) was among the first to demonstrate that the growth of a fungus renders the medium unfavorable to the subsequent growth of the same organism Species of *Peziza* and *Aspergillus* have an antagonistic effect upon one another, which, according to Reinhardt (273), is a result of the production of acid, chiefly oxalic Nikitinsky (238) suggested that the inhibiting effects are due to unfavorable changes in reaction Culture solutions in which fungi have grown are not suitable for the germination of freshly inoculated spores and are improved by boiling (188), not only the same organisms, but also other species are checked in their growth (32)

Eijkman (88) demonstrated that bacteria produce in the medium thermolabile toxic substances, when such a medium is heated, it is again made suitable for bacterial development, the fact that growth is not as good as in fresh medium was explained as due either to exhaustion of some of the nutrients or to the formation of injurious substances Bacterial spores are able to germinate again in the same medium, if this is boiled Certain bacterial metabolic products, even when heated to 120°, have a strong influence upon the growth of various microorganisms (228)

According to A J Brown (37), staling of a culture is due not to the accumulation of metabolic products but to the exhaustion of substances which stimulate growth, as in the case of oxygen for yeast Pratt (263) concluded that exhaustion of food is not a primary factor in staling, the latter is due largely to the formation of bicarbonate (39, 125) This effect is partially corrected by boiling and by adjustment of the reaction, treatment with ether removes the residual staleness, colloidal clay and charcoal also remove this effect The phenomenon of staling was sometimes spoken of as "vaccination" of the medium (14, 275), and believed to be due to protein degradation products

Fungi may produce (188, 202) not only growth-inhibiting but also growth-promoting substances; by means of certain procedures, it is possible to separate one from the other (244). The tendency of fungus hyphae to turn away from the region in which other hyphae of the same fungus are growing (negative chemotropic reaction) has been explained (111, 135) as a response to chemical substances produced by the growing fungus.

The repressive type of antagonism results in a delay in the growth of the antagonized organism (231, 109). The bactericidal type results in the destruction by the antagonist of another organism without producing any lytic effect, *Bacillus mesentericus* (*vulgatus*) being able not only to depress but also to kill diphtheria and pseudo-diphtheria bacteria (373). The production by an antagonist of metabolic products which possess lytic properties and which modify the growth of various bacteria has been designated as "direct antagonism." On the other hand, "indirect" or "passive antagonism" has been looked upon (231) as depending not upon the direct action of the antagonist but upon changed conditions of culture which become unfavorable for the particular organism (150); here belong changes of pH and rH values, and the impoverishment of nutrients in medium. Neufeld and Kuhn (236) limited direct antagonism to those phenomena where the action is due to the living cell itself, as in the repression of anthrax bacilli by intestinal bacteria (142).

Bail (11) suggested that there exists, for every bacterium, a typical constant number of living cells capable of living in a given space. When this concentration (M) is reached, multiplication comes to a standstill without the nutrients being exhausted or toxic substances produced. The same is believed to hold true when two bacteria live together (363). If the limiting concentrations of the two organisms are different, the one with a higher M value will repress the other; the weaker species may check the stronger one when planted in a sufficient excess (98). It has been suggested (144) that "biological activity" and "competitive capacity" must also be taken into consideration.

Antagonism may be either one-sided or two-sided, namely, when only one bacterium represses another, which is not antagon-

istic to it, or when each organism represses the other (115) One-sided antagonism may become two-sided under certain conditions of culture *E coli*, for example, is antagonistic to *E typhosa*, however, if the latter is inoculated into a medium somewhat earlier than the former, the reverse is true (343, 122) Although antagonistic effects have usually been observed for one species of bacteria against another, very often one strain of the same species may exert antagonistic effects upon another strain (144, 221) Non-flagellated variants of typhoid bacteria are repressed by a flagellated form, smooth variants of paratyphoid bacteria by rough variants, etc The fact that all bacterial cultures stop growing after a certain period of time has also been interpreted as a result of antagonism of some cells upon others When the filtrates of such cultures are added to fresh nutrient media they may stop the growth of the same species as well as that of other species Rahn (268) observed that the phenomenon of iso-antagonism is associated with the formation of a thermolabile substance, not passing through a filter This substance is often destroyed by light (88)

Among the various types of antagonism, the most definite and the one which is best understood is that which results in the formation of antagonistic substances The nature of these substances or toxins when produced by different bacteria and fungi is not always the same Some are destroyed by boiling, by exposure to light or by filtration (202), others are resistant to heat and to ultraviolet rays, some are readily adsorbed by filters, from which they can be removed by special solvents

The abundance of antagonistic substances produced by many fungi and bacteria is (97) greatly influenced by the energy and nitrogen sources in the medium Schiller (300, 301) believed that antagonism could be induced by withholding certain nutrients in a dilute glucose solution without nitrogen, yeasts were said to be "forced" to kill and digest bacteria, if a few loops of yeast suspension were added to a fully developed bacterial culture, the yeasts produced a bacteriolytic substance which was also active outside of their cells On the other hand, various bacteria killed the yeasts when inoculated into cultures of the latter sus-

pended in distilled water The destruction of the fungus *Ophiobolus*, causing the take-all disease of cereals, by soil organisms is believed to take place in a similar manner (116)

It has often been observed that certain organisms produce pigments in the presence of others, and that these pigments are in some way associated with the phenomenon of antagonism *V cholerae* produces, in the presence of *Sarcina lutea*, a dark violet pigment which is accompanied by an increase in agglutination and virulence (159, 278a) The destruction of *Dictyostelium mucoroides* by a red-pigment-producing bacterium is accompanied by intense pigmentation (258), the blue pigment of *Bacterium violaceum*, however, only delays the growth of the fungus *Penicillium africanum* (72) produces a more intense pigment in contact with other fungi, such as *Aspergillus niger*, this pigment accumulates in the mycelium of the latter, which may thereby be killed Nadson (229) demonstrated that the pigment of *Penicillium luteum* or *Spicaria purpurogenes* is used not only for purposes of protection, but also for attack upon other organisms, whereby the latter are killed and stained

The various theories concerning the mechanism of antagonism may be summarized (231) as follows

1 Exhaustion of nutrients in medium (249, 250, 109, 121)

2 Physico-chemical changes produced by the growing organism in the medium, including changes in osmotic pressure, surface tension, oxidation-reduction potential and reaction (8, 307, 109, 22, 28, 210, 334)

3 Production of specific enzymes, either by the antagonist itself or as a result of autolysis of the antagonized cells

4 Production and liberation of specific substances, which have a selective bacteriostatic and bactericidal effect, or fungistatic and fungicidal action (36, 115, 109, 93, 77, 348, 104, 356)

5 Certain types of reactions, which may be designated as action at a distance (3, 253)

6 Space antagonism

A great number of methods have been developed for measuring antagonistic action (109, 115, 314, 110, 265, 193, 213)

Porter (260) recognized that different organisms exhibit vary-

ing degrees of inhibition as well as different mechanisms of inhibition Often one organism may completely check the growth of another, later, growth may be resumed, although not quite normally The morphological effects produced by antagonists comprise changes in form, size and structure of hyphae, direction of growth, as well as complete cessation of growth and abbreviation of hyphal segments Among bacteria, the spore-formers are strong inhibitors, and actinomycetes exhibit strong inhibitory action against most filamentous fungi Phycomycetes and basidiomycetes are more or less inert Ascomycetes and Fungi Imperfecti vary greatly in their inhibitory action Some yeasts are strong inhibitors

Very little is known of the defense mechanisms of microorganisms against the effect of toxic substances produced by antagonists Green (137) has shown that extracts of *Brucella abortus* and other bacteria contain a factor, designated as "P", which specifically inhibits the bacteriostatic action of sulphanilamide This substance stimulates the growth of many bacteria The neutralization of a growth inhibitor of bacteria by a growth stimulant has been indicated for *p*-aminobenzoic acid against sulphanilamide (289) The ability of many bacteria to produce an enzyme which destroys the bactericidal agent of microorganisms has been demonstrated in the case of penicillin (2)

ANTAGONISTIC EFFECTS OF BACTERIA

Among the bacteria most frequently mentioned as possessing strong antagonism to pathogenic organisms, *Pseudomonas fluorescens*, *P putida* and *P aeruginosa* (*Bacterium pyocyaneum*) occupy, in the early literature, a prominent place Garré (115) found that *P putida* inhibits the growth of *Staphylococcus aureus*, *E typhosa* and *Bacterium mucosus-capsulatus*, but not of *Bacillus anthracis* and others Lewek (192) soon reported that *B anthracis* is also killed by the above antagonist, whereas the growth of *S aureus* and *Vibrio comma* is only retarded, and no effect at all is exerted upon *E typhosa* and *E coli*, *P fluorescens* is antagonistic to *B anthracis*, but not to other organisms However, Ohitski (245) demonstrated, in 1891, that *P fluorescens*

inhibits the growth not only of *E typhosa*, but also of *B anthracis*, *V comma*, *Serratia marcescens* and *S aureus* These and other apparently contradictory results were probably due to the specificity of the strains used by different investigators

According to Laws and Andrews (190), the presence of *P fluorescens* in sewage greatly reduces the period of survival of the typhoid organism Horrocks (160) also found that the latter does not develop in gelatin upon which *P fluorescens* was previously grown The pathogen could not be detected in sterile sewage after the antagonist was present for seven days An antagonistic effect against *E typhosa* was also exerted by *E coli*

Frost (110) established, in 1901, that a number of different bacteria are able to exert a marked antagonism against *E typhosa* *P fluorescens* exhibited the strongest effect, *Proteus vulgaris* acted more rapidly, but the active substance did not diffuse to so great a distance into the medium Filterable and thermostable antagonistic substances were produced, their action varied with temperature, being most pronounced at 37°, at ice-chest temperature, the action was so delayed that the pathogen had an opportunity to develop This was believed by Frost to offer a possible explanation of the fact that when water supplies become contaminated in cold weather their power of producing infection is retained longer than when the contamination takes place in warm weather

The activity of the influenza organism is largely dependent on the presence of accompanying bacteria (365), some of these, especially micrococci, are favorable to its growth, whereas others, such as *P aeruginosa* and *B subtilis*, are injurious Lewis (193) observed that the luxurious growth of *P fluorescens* in manured soil and in protein solution containing *B cereus* is due to the antagonism of the former against the latter *B anthracis*, *B megatherium*, *Vibrio comma*, *Chromobacterium violaceum* and *Rhodococcus* were also inhibited, *Salmonella* species were less sensitive, whereas *E coli*, *Aerobacter aerogenes* and *Serratia marcescens* were highly resistant *P fluorescens* was found to produce a water-soluble, thermostable substance which was toxic to various bacteria, except the green fluorescent forms, it was also active against actinomycetes but not against fungi

Spore-forming bacteria as antagonists

Among the spore-forming bacteria, *Bacillus subtilis*, *B mycoides*, *B mesentericus*, and, to a lesser extent, *B brevis* and *B simplex*, as well as some of the more heat-resistant types, the so-called *Tyrothrix*, occupy a prominent place as antagonists, as shown in table 1

TABLE 1

Spore-forming bacteria as antagonists

ANTAGONIST	ORGANISMS AFFECTED	KNOWN PROPERTY	REFERENCE
Bacillus anthracis	Anthrax, typhoid and lactic acid bacteria		307, 293, 109
B brevis	Gram-positive bacteria	Two substances crystallized	77
B mesentericus	Typhoid, anthrax, Shiga, pneumococci	Bacteriolytic	237
	Many bacteria		152
	Diphtheria bacteria	Bactericidal	10, 352
	Helminthosporium sativum		55, 260
B mesentericus vulgatus	*C diphtheriae*	Thermolabile, non-filterable	265
B mycoides	7 to 20 species of bacteria	Lysis	225, 226
	M tuberculosis	Thermostable, precipitated by tungstic acid	176, 177
	Helminthosporium teres		260
B mycoides, var *cytolyticus*	Most pathogenic bacteria and many non-pathogens		107
B simplex	*Rhizoctonia solani*	Thermostable	60
B subtilis	Various bacteria	Bacteriolytic	237
	M tuberculosis, *E typhosa*		337, 18
	Rabies virus		206
B subtilis-mesentericus	Mostly living gram-positive and dead gram-negative bacteria	Lysis	286, 287
Spore-forming bacteria	Fungi		270, 90, 12, 273, 260

The term "lysobacteria" was applied (286) to those capable of dissolving living and dead organisms The following differences were recognized between the action of antagonists and that of phage (*a*) the filtrate of the antagonist is active against other

bacteria, fresh filtrates of the antagonists are most active, the activity being destroyed at 70°, (*b*) both living and dead cultures of the antagonized bacteria are dissolved, (*c*) the action is not so specific as in the case of phage, (*d*) races of *E coli* resistant to phage are dissolved by the filtrate of the antagonist The filtrate acts upon intestinal bacteria not only *in vitro* but also *in vivo*

Spore-formers are active antagonists against diphtheria and pseudodiphtheria organisms (10) Since these antagonists were not found in saliva, and the saliva bacteria were not active, the conclusion was reached that the action of saliva against bacteria was due to another factor rather than to its bacterial content Dubos (77) isolated from a soil enriched with various living bacteria a gram-negative organism (*Bacillus brevis*) which has a marked lytic effect against gram-positive bacteria, including staphylococci, pneumococci, and others An active substance was isolated which also acts upon these bacteria *in vitro* and *in vivo* Hoogerheide (157) also isolated from the soil an aerobic, spore-forming bacillus which produces a very active substance it prevents the formation of capsules by Friedlander's bacterium and is highly bactericidal Strains of spore-forming bacteria producing antagonistic substances are widely distributed in the soil, they are non-diastatic, gram-negative and produce hydrogen sulfide (320)

Spore-forming bacteria are found to produce substances antagonistic not only to bacteria but also to fungi (table 1) Cordon and Haenseler (60) isolated a spore-forming bacterium (*Bacterium simplex*) which is antagonistic to *Rhizoctonia solani*, an important plant pathogen, the bacterium produces a thermostable substance, which inhibits the growth and even causes the death of the pathogen *B mesentericus* grown on artificial media produces an active substance, which suppresses the growth of *Helminthosporium sativum* (55) It increases sporulation, inhibits or retards spore germination, causes abnormal hyphal growth, and induces mutations in certain strains of the fungus The substance is thermostable, diffusible, passes through a Berkefeld filter, is adsorbed by infusorial earth, withstands freezing and desiccation, and does not deteriorate readily It is destroyed by alkalis

but not by acids, and is inactivated or destroyed by certain fungi and bacteria.

The antagonistic spore-forming bacteria produce substances which act primarily upon gram-positive bacteria, but also to some extent upon gram-negative organisms. It is of particular interest to note that living gram-positive bacteria are more susceptible to the action of these antagonists than living gram-negative bacteria, whereas the reverse is true in the case of dead organisms (287).

Non-spore-forming bacteria as antagonists

Since the early work of Bouchard (31), Emmerich and Löw (93) and others, numerous non-spore-forming bacteria have been shown (155) to be able to antagonize other bacteria (table 2), in many cases, the active substance was isolated and its chemical nature determined. Particular attention was paid to the pyocyaneus and fluorescens groups, and much consideration was also given to the members of the colon-typhoid group.

Wathelet (350) found that, in mixed culture, the colon organism gradually replaces the typhoid (60, 239, 148, 302, 180, 224). Chatterjee (51) noted that typhoid and paratyphoid bacteria fail to multiply when inoculated into media in which the colon bacterium has previously grown, a fact also reported by various other investigators (340, 209, 36, 343, 70, 254, 264, 370, 255, 324, 180). The antagonistic action of paratyphoid against typhoid bacteria has also been established (173). Nissle (239) introduced the term "antagonistic index" to express the relation between typhoid and colon organisms in a culture of the former inoculated with the latter. The term "minimum inhibitory ratio" was used to designate the ratio between two species at which one will overgrow the other (98, 236). Fulton (112) noted that when *E. coli* and *Salmonella schottmülleri* are grown in association, the second is at first inhibited, but, after *E. coli* passes its maximum development, it also makes a good growth. The occurrence of slow lactose-fermenting strains of *E. coli* in stools (168), as well as the inhibitory action found in certain stools seeded with *E. typhosa* was ascribed to the antagonistic action of the former (239). Different strains of *E. coli* appear to repress the typhoid organism

TABLE 2

Non-spore-forming bacteria as antagonists

ANTAGONIST	ORGANISMS AFFECTED	KNOWN PROPERTY	REFERENCE
Achromobacter sp	Species of *Fusarium*, *Sclerotinia*, *Botrytis*		56
Aerobacter aerogenes	*B anthracis*, *Pasteurella pestis*		142, 100
Alcaligenes faecalis	*Helminthosporium* sp		260
Anaerobic bacteria	*M tuberculosis*, *B anthracis*		248, 155
Diplococci and pneumococci	Various bacteria	Thermolabile	199, 269, 247, 200, 100, 81, 165, 98, 144, 236
Eberthella typhosa	*E typhosa*, *P fluorescens*, *E coli*, *B anthracis*		115, 343, 131, 335, 305, 85
Escherichia coli	Typhoid, paratyphoid, diphtheria, staphylococci	Mostly growth inhibition	350, 239, 51, 18, 143, 180, 271
	Proteolytic and putrefactive bacteria		19, 329, 279
	M tuberculosis, *B anthracis*, and spore-forming bacteria		142, 126, 166, 306, 293, 335, 45, 172
E coli strains	Other *E coli* strains		239
Klebsiella pneumoniae	Anthrax, diphtheria, bubonic plague, etc	Active filtrate	252, 305, 109, 19, 236, 18, 100
Micrococci	*V comma*, *M tuberculosis*, *E typhosa*, *B melitensis*, etc		199, 81, 231
Myxobacteria	Plant-disease-producing bacteria	Thermostable, lytic	312
Pasteurella avicida	*B anthracis*, *E typhosa*		250, 109
Proteus vulgaris	*B anthracis*, *P pestis*		335, 100
	Clostridium sporogenes and other anaerobes		242, 13, 353, 156
	Phytophthora, *Basisporium*, *Sclerotium*, *Glomerula*		174, 260
Pseudomonas aeruginosa	*B anthracis*, *E typhosa* *V comma*, etc	Thermostable, filterable	31, 50, 23, 109, 93
	Gram-negative bacteria, *M tuberculosis*, and yeasts	Depressed growth	280, 31, 369, 23, 138, 281

TABLE 2—*Concluded*

ANTAGONIST	ORGANISMS AFFECTED	KNOWN PROPERTY	REFERENCE
P fluorescens	*E coli*, *S marcescens*, *C diphtheriae*, *B anthracis*, etc	Thermostable, filterable	115, 150, 307, 192, 245, 160, 110, 193, 100, 288
	Actinomycetes	Lytic action	211
P phaseoli	*Fusarium* sp		21
P translucens	*Ophiobolus graminis*		34
Salmonella paratyphi	*E coli*, *B anthracis*, *P pestis*		168, 335, 100
Serratia marcescens	*C chauvoei*, *B anthracis*, staphylococci, micrococci, fungi	Non-pigmented, thermostable, lytic	80, 277, 282, 89, 208, 16
	Gram-positive but not gram-negative bacteria	Alcohol-soluble pigment	150
Staphylococci	Dead cells of various bacteria		134, 366, 367
	Gram-positive bacteria, anthrax, diphtheria, plague organism		101, 18, 82, 100, 61
Streptococci	Anthrax, diphtheria, etc	Antagonism not associated with hemolytic properties or virulence	73, 91, 252, 109, 231, 302, 18, 57, 45
Streptococcus cremoris	*S lactis*		362
S lactis	*L bulgaricus*	Thermostable, non-filterable	283
S muscosus	*P pestis*		100
S pyogenes	*B anthracis*, *Phytomonas tumefaciens*		25

to a different extent, freshly isolated strains being more active than stock cultures (310) However, young, actively growing cultures of *E typhosa* inhibit the growth of *E coli*, older cultures being non-antagonistic (343)

A bacteriophage was found (197) to develop as a result of the antagonistic action of *E coli* against the Shiga bacillus, this was believed to occur in the intestines where antagonistic conditions are always present A similar antagonistic stimulus was observed (99) for *Salmonella albus* to *E coli* The weakest antagonists

were said (148) to belong to the paracolon group, the strains of medium activity to the colon organism, and the strongest antagonists to the *E coli-immobilis* type

Gratia (129 to 131) demonstrated that one strain of *E coli* may be inhibited by another (67), however, some cells of the former may remain immune against the action of the latter An emulsion of dead cells may become clear, when living cells are added, an effect that was designated as *autophage* The mechanism of this action was variously explained by a change in pH value of the medium, oxidation-reduction potential, or some direct effect of the bacteria Thermolabile, filterable substances have frequently been demonstrated (59, 142, 205) These were considered either as auto-toxins or as proteolytic enzymes (255) Schilling and Califano (302) found that the filtrate of *E coli* depressed only the dysentery organism of Shiga The active substances produced by *E coli* were believed (145) to be thermostable lipoids, which are capable of bringing about lysis of the colon and other bacteria

An extensive literature has also accumulated on the antagonistic action of cocci Holman (156) suggested that many chances of error are possible in the case of mixed cultures, particularly with closely similar forms, pneumococci, for example, were found to be able to live for long periods with non-hemolytic streptococci Peculiar antagonistic relations between pneumococci and staphylococci were also observed (6) It was suggested (11) that adaptive alterations are to be expected in the growth of bacteria in mixed cultures, the antagonism of one or the other was believed to depend frequently upon their relative numerical abundance (98)

Lactobacillus bulgaricus was found able to modify the variation of *E coli* from the *S* to the *R* phase, inhibiting its development and even bringing about its lysis, this took place only in the presence of the living antagonist, no active substance could be demonstrated, and lactic acid itself had only a limited effect (5) When a yellow sarcina was used as the "feeding organism," a stimulating effect was exerted on the growth of *Brucella* sp on solid media, in liquid media, however, the life activity of the latter was repressed (182) A white staphylococcus exerted an antagonistic action on *Brucella* sp both in liquid and on solid media

Certain acid-producing aerobes were found to inhibit toxin production by *Clostridium botulinum* in glucose but not in non-carbohydrate media (146) Since acid itself was ineffective, Holman (156) suggested that the acid must be in a nascent state A mixture of a *Clostridium sporogenes* with *C botulinum* interfered with the development of the toxin by the latter, it was thought possible that this association might even cause the early disappearance of the botulinus toxin (170, 63, 106)

The antagonistic action of the non-spore-forming bacteria comprising a great variety of organisms is no doubt due to several agents and mechanisms Very few of these are as yet sufficiently understood, although many attempts have been made to utilize these antagonisms for disease control

Antagonistic effects of actinomycetes

The ability of actinomycetes to repress the development of other microörganisms appears to be widespread (table 3) In view of the difficulty of identifying these organisms as specific, well recognized types, most of the references are either to "white" or "pigment-producing" types, or just to plain "Actinomyces" Since there are hundreds of species now recognized and not all of them possess antagonistic properties (230), the identity of the antagonists may be considered, in most cases, to be unrecognized This makes a comparison of the results of different investigators rather difficult

In 1890, Gasperini (119) demonstrated that certain species of *Actinomyces* have a marked lytic effect upon bacteria and fungi Greig-Smith (140, 141), in his studies on the presence of toxic substances in soil, found that the antagonistic action of actinomycetes was directed against many bacteria as well as against certain fungi, the fact that they grow only slowly in normal soils suggested the possibility that they comprise an important factor limiting bacterial development In an attempt to find organisms that are effective against diphtheria of the pharynx, Rosenthal (285) succeeded in isolating from the air a species of *Actinomyces* which he designated as the true biological antagonist of Loeffler's organism The surface of an agar plate was covered with an emulsion of diphtheria bacteria and inoculated in several spots with the antagonist

At the end of two days, the plate was covered with the growth of the diphtheria organism, except that the colonies of the actinomyces were surrounded by large transparent zones

Gratia and Dath (133) suspended dead cells of staphylococci and other bacteria in agar and exposed the plates to the air A white species of *Actinomyces* developed on the plates When this organism was transferred to a suspension of dead staphylococci in sterile saline, characteristic flaky growth was produced and the bacterial suspension became clarified in 36 hours When the lysed emulsion was filtered, it could again dissolve a fresh suspension of the dead bacteria This species of *Actinomyces* was found to be able to attack all staphylococci tested, as well as certain other bacteria such as *P aeruginosa*, however, it was inactive towards *M tuberculosis* and *E coli* This phenomenon was believed to resemble the "induced microbial antagonism" of Schiller (301), but it was distinct from bacterio-

TABLE 3

Antagonistic action of actinomycetes

ANTAGONIST	ORGANISMS AFFECTED	KNOWN PROPERTY	REFERENCE
Actinomyces antibioticus	All bacteria and fungi, especially gram-positive types	Thermostable, largely bacteriostatic	348
A praecox	*A scabies*		219, 220
Actinomyces sp	Bacteria and fungi	Lytic action	119
Actinomyces sp	Diphtheria	Growth inhibition	285
Actinomyces sp	Pneumococci, streptococci, staphylococci, *P aeruginosa*	Lysis of dead cells Substance thermolabile	131, 133 359
Actinomyces sp	*B mycoides*, proactinomycetes, mycobacteria	Bactericidal, with or without lysis	30, 185
Actinomycetes	Dead and living bacteria	Lysis	195
Actinomycetes	Spore-forming bacteria	Repression of growth	193
Actinomycetes	Gram-positive bacteria	Thermostable, produced on synthetic media, resembles lysozyme	230, 346, 186
Actinomycetes	*Pythium*	Thermostable	328

phage This type of antagonism was considered to be widely distributed in nature and directed against many bacteria, pathogenic and saprophytic The fairly stable, active substance was present extensively in old cultures of this actinomyces, and was regarded as a highly specific proteolytic enzyme Some strains of the actinomyces could also attack *E coli*, but this property could be lost The lysed material was designated as a "mycolysate", it did not possess the toxicity of the non-lysed suspension, but retained the antigenic properties of the latter (132) Gratia later (131) asserted that the actinomyces was also able to attack living cells of bacteria, except *E coli* and *E typhosa* which had to be first killed by heat

Welsch (359) designated this bacteriolytic substance as *actinomycetin* Some of the activity was lost on passage through bacterial filters Welsch divided the bacteria in their relation to actinomycetes into three groups (1) those organisms which are lysed by the aqueous extract of agar cultures, namely pneumococci and hemolytic streptococci, (2) bacteria which are not dissolved even by the most active soluble substance, but which are depressed by the mycelium of the actinomycetes, including various sarcinae and *B megatherium*, (3) bacteria not acted upon either by the mycelium or by the active substance, comprising the colon-typhoid-paratyphoid and the pyocyaneus groups When these bacteria are killed by heat or are placed under conditions unfavorable to multiplication, however, they are dissolved by the lytic substance Cells of *E coli* acted upon by radium emanation, which stops their multiplication, become susceptible to the lytic substance

Various actinomycetes are reported (30) able to repress and lyse living cells of spore-forming bacteria A thermostable toxic substance is produced, especially on agar media The action of the toxin is weakened by an alkaline reaction and favored by an acid reaction When *B mycoides* and an antagonistic actinomyces were inoculated together in peptone media, no toxic action was exerted, because the former alkalinized the medium rapidly, thus making conditions unfavorable for the production of the toxin by the antagonist The action of the toxin on *B mycoides*

resulted in elongation of the vegetative cells, due to a delay in fission and suppression of sporulation According to McCormack (211), aerobic conditions are necessary for the development of the antagonistic properties of actinomycetes, those requiring less oxidized conditions are themselves antagonized *B megatherium* was said to be antagonistic to certain species of *Actinomyces* but was itself antagonized by others

Many species of actinomycetes (but not proactinomycetes) were found (185) to produce a substance which possesses a strong bactericidal action against various microorganisms, including proactinomycetes, mycobacteria and micrococci The cells were either lysed, or killed without subsequent lysis Spore-bearing bacteria were not killed but were stopped in their development, however, non-spore-forming bacteria, including nodule bacteria and *Azotobacter* sp , were not affected and actually grew in the filtrates of the antagonists

Antagonistic actinomycetes are widely distributed in the soil (230) Out of 80 cultures isolated, 47 possessed antagonistic properties, but only 27 produced toxins These acted upon the same gram-positive bacteria, but not upon gram-negative bacteria or fungi No relation was observed between active antagonism and pigmentation of the colonies, formation of soluble pigments (346), manner of sporulation or shape of spores Some strains were able (230) to excrete water-soluble toxic substances into the medium but others did not The substances were thermostable heating for 30 minutes at 1 5 atm only reduced somewhat their activity Since the capacity to produce antagonistic substances was possessed only by certain species, the utilization of this phenomenon for the systematization of actinomycetes as a whole was suggested Based upon the action of the antagonistic substance, mycobacteria could be differentiated from non-spore-forming, especially nodule, bacteria The production of the active substance was highest on synthetic media, and was rather weak or even totally absent in protein media

Waksman and Woodruff (348) isolated from the soil an organism, described as *Actinomyces antibioticus*, which proved to be particularly active against a great variety of bacteria and fungi

It produced a highly bacteriostatic substance designated as *actinomycin*. The organism was aerobic and produced dark-brown to black pigments on protein- and peptone-containing media, however, it was distinct in its physiology from the other chromogenic species.

Millard (219, 220) succeeded in controlling potato scab, caused by *Actinomyces scabies*, by the use of green manures and grass cuttings. The development of scab on potatoes grown in sterilized soil inoculated with *A. scabies* could be reduced by the simultaneous inoculation of the soil with *A. praecox*, an obligate saprophyte. By increasing the proportion of the latter to the pathogen, the degree of scabbing on the test potatoes was reduced from 100 per cent to nil. According to Goss (128), the general soil microflora has a controlling effect upon the development of scab, inoculation with *A. praecox* alone gave negative results. Sanford (294) was also unable to obtain control of potato scab by the inoculation of both steam-sterilized and natural soil containing different amounts of green plant materials with *A. scabies* and *A. praecox*. These organisms were perfectly compatible on potato-dextrose agar, as well as in a steam-sterilized soil medium. It was suggested that the control of scab obtained by Millard was possibly due not to the direct action of *A. praecox* but to certain other undetermined microorganisms favored by the presence of the green manure, or by other conditions (175).

ANTAGONISTIC EFFECTS OF FUNGI

A most extensive literature has accumulated on the antagonistic effects of fungi, especially from the point of view of causation of plant diseases. The various interrelationships studied involve the action of (*a*) fungi against bacteria, (*b*) of fungi against fungi, and (*c*) of bacteria against fungi (table 4).

In the study of staphylococcus variants, Fleming (104) observed that around a large colony of a contaminating fungus, which proved to be *Penicillium notatum*, the staphylococcus colonies became transparent and were obviously undergoing lysis. The pure culture of the fungus had marked inhibitory, bactericidal and bacteriolytic properties for many of the more common

pathogenic bacteria, including staphylococci, streptococci, diphtheria bacilli, gonococci and meningococci, but not for the organ-

TABLE 4

Antagonistic effects of fungi

ANTAGONIST	ORGANISMS AFFECTED	KNOWN PROPERTY	REFERENCE
Cephalothecium roseum	*Helminthosporium sativum*		136
Fungi	Fungi	Antagonism against the same or other species	89, 317, 274, 111, 360, 318, 260, 355, 64, 54, 240, 86, 304, 342
Helminthosporium sativum	*Ophiobolus graminis*	Thermostable	35
Helminthosporium sp	Various fungi		260
Hyphomycetes	*Pythium*		75
Penicillium luteum	*Citromyces*, *Aspergillus niger*	Thermostable	351, 246
P notatum	Staphylococci, streptococci, diphtheria, gonococci, meningococci, anaerobic bacteria, etc	Lytic action	104, 58, 272, 48, 29
Penicillium sp	Various fungi	Thermostable	35, 273
Penicillium sp	*Helminthosporium sativum*	Suppression of growth	297
Psalliota campestris	*Mycogone*		53
Torula suganii	Various fungi		244
Torulopsis sp	*Dematiaceae*	Inhibition of growth	215
Trichoderma lignorum	*Fusarium*, other fungi		124, 7
	Actinomyces scabies		65
Trichoderma, *Gliocladium*	*Rhizoctonia*, *Pythium*, *Phytophthora*, *Fusarium*, *Rhizopus*, *Sclerotium*, *Blastomycoides dermatitis*, other fungi	Lethal principle isolated	7, 260, 149, 354

isms of the colon-typhoid-dysentery group The filtrate of the culture contained the active substance which was designated as *penicillin* (58, 272)

Chain *et al* (48) succeeded in obtaining from the culture medium a water-soluble, stable, brown powder of penicillin which had great anti-bacterial activity The impure substance inhibited, in dilutions of 1 part in several hundred thousand, the growth of many aerobic as well as anaerobic bacteria (*C welchii*, *C septicum*, and *C oedematiens*) The material was also active *in vivo*, subcutaneous injections saving the lives of mice injected intraperitoneally with *Streptococcus pyogenes* or *Staphylococcus aureus* Intramuscular infections of mice with *C septicum* were also successfully treated by repeated subcutaneous injections of penicillin Bornstein (29) found that twenty-seven strains of enterococci and six of *Streptococcus lactis* were resistant to penicillin, thirteen strains of *S viridans* were susceptible Other fungi also appear capable of producing bactericidal substances (361)

Harder (147), in a study of the behavior in mixed culture of fungi belonging to the Basidiomycetes and Ascomycetes, found that young colonies do not produce so much of the toxic substance as do older colonies, hence they can grow close to one another *Coniophora cerebella* was held back by a species of *Penicillium*, its mycelium being considerably modified, in time, the former organism adapted itself to the latter and overgrew it at a rate eventually more rapid than that of a pure culture

In some cases, as for example, the reciprocal influences of *Sclerotium rolfsii* and *Fusarium vasinfectum*, it was found (284) that, at pH values below 6 9, the former completely overgrew the latter, whereas in alkaline ranges the reverse process took place Several species of *Penicillium* and other soil-inhabiting fungi were tested in steam-sterilized soil for their effects on the virulence of *Helminthosporium sativum* for wheat seedlings (297) Certain of the fungi exerted a marked degree of suppression, some had no effect, while others increased the virulence of the pathogen There were marked variations in activity among the species of *Penicillium*

Certain fungi commonly found in the soil (species of *Trichoderma*, later recognized as *Gliocladium*) have a decided inhibiting effect against various fungi, such as *Rhizoctonia* and other plant pathogens (354) This effect is due to the production of an active

substance in the filtrate of the antagonist This "lethal principle," designated as *gliotoxin*, kills the antagonized fungus, it is also found to be highly toxic to *Blastomycoides dermatitis*, a causative agent of human skin disease

The fungicidal effect of many bacteria has also been definitely established Bamberg (12) isolated from corn plants certain bacteria which inhibited the development of *Ustilago zeae* and destroyed the colonies of this fungus It was believed that the widespread distribution of these bacteria might bring about a check on the multiplication of the pathogenic fungi in the soil. Four types of bacteria antibiotic to smuts and to certain other fungi were described by Johnson (169), some of the bacteria were found to produce enzymes that were able to dissolve the cell walls of the fungus sporidia Carter (46) found that *Helminthosporium sativum* and a certain unnamed bacterium produce thermostable mutually inhibiting substances, the bacterium and its products inhibited the growth of this fungus as well as of other members of the same genus The inhibitory agent was diffusible, acting upon *H sativum* on potato-dextrose plates for a distance of 10 to 15 mm The toxicity of *B mesentericus* for this fungus has also been established (55)

Two bacteria belonging to the genera *Pseudomonas* and *Achromobacter* have been isolated (56) and found capable of bringing about the lysis of different species of *Fusarium* and other fungi These bacteria were widely distributed in the soil, but were absent in certain flax-sick soils, in spite of the abundance of *Fusarium* sp The fungus did not develop and the plant disease did not occur in the presence of the active bacteria Nakhimovskaia (232) found that the presence of certain bacteria (*P fluorescens*, *Serratia* sp) in the medium inhibits the germination of rust spores, spore-forming bacteria and sarcinae did not exert any antagonistic action, but their presence affected the nature of the germination process, the spores giving rise to mycelium-like forms with great numbers of copulating filaments, whereas, in the control cultures, yeast-like forms prevailed and copulating cells were rarely encountered

In the infection of wheat seedlings by *Ophiobolus graminis*, a

number of fungi and bacteria were found (296) to exert a marked antagonistic action, not only the living cultures, but the culture filtrates were also effective in many cases (96) The growth of *H sativum* and *Fusarium graminearum* upon sterilized soil could be completely suppressed by the addition of small amounts of unsterilized soil or by the simultaneous inoculation with a number of other fungi and bacteria, so that no infection resulted when wheat seeds were inoculated with this soil (149) The fact that root-rot diseases of wheat are less severe when the crop is grown on summer-fallowed land than on land cropped to wheat for several years could be related to the growth of soil saprophytes, which in bare fallow have an advantage over the pathogens in competition for food Infection of wheat seedlings by *O graminis* in sterile soil fell off rapidly with the reëstablishment of the original soil microflora (34) An inverse correlation was reported (223) between the degree of infection and the protective effect of the general soil microflora An increase in soil temperature was found to increase the antagonistic action of the soil microflora to the parasitic fungus (149, 117)

The decay of fruits can also be suppressed or modified by inoculation with mixtures of known organisms (299) According to Potter (262), *Pseudomonas destructans,* the cause of rot of turnip, produces a potent, heat-resistant plant toxin, which is also destructive to the pathogen itself By spraying turnips with this bacterial product, the disease could be checked The same principle was found to hold true for oranges infected with *Penicillium italicum* The injurious action of certain common soil bacteria on *Pseudomonas citri,* the cause of citrus canker, has also been reported (111) Wheat seedlings were protected from infection by Helminthosporium and flax seedlings from Fusarium by the use of antagonistic bacteria (260) A watermelon disease caused by *Phymatotrichum omnivorum* was reduced when certain fungi (*Trichoderma lignorum*) and bacteria were present in the soil together with the pathogen (38) The severity of the seedling blight of flax, caused by *F lini,* was diminished when the pathogen was accompanied in the soil by certain other fungi (325) The pathogenicity of *H sativum* on wheat seedlings was suppressed by

the antagonistic action of *Trichothecium roseum*, which is believed to produce a toxic substance (136)

The role of microbiological antagonism in the natural control of soil-borne fungus diseases of plants has been emphasized (295, 33, 296, 149, 34, 21) The principles of biological control have been outlined (118) as follows The soil population is in a dynamic biological equilibrium When a certain crop is grown continuously, the multiplication of various parasites capable of attacking the roots of that crop takes place (27) Organic manures are known to stimulate the development of various saprophytes in the soil These multiply at the expense of the pathogens and are able to check their activity, either by preventing their growth (*fungistatic* action), or by attacking and destroying the mycelium of the parasites (*fungicidal* action) The biological control of plant diseases is said to be most effective against those organisms which have become highly specialized to a parasitic form of life

Van Luijk (341) suggested that biological control of plant parasites may be obtained by inoculation of the soil with specific microörganisms selected for their antagonistic capacity, or by the addition of their growth products However, Broadfoot (34) emphasized that the antagonism of a saprophyte to a plant pathogen, as measured by growth on artificial media, is not a reliable measure of the actual control that may be exerted upon the parasite in the soil A lack of specific microorganisms is not considered to be a sufficient factor limiting biological control under natural conditions No inoculation of soil with an antagonistic organism, such as *T lignorum*, can have more than a temporary effect in changing the microbiological balance of the soil population Similar results were obtained by Weindling and Fawcett (357, 101), in their attempts to control *Rhizoctonia solani* by the use of *T lignorum*, and by Cordon and Haenseler (60), by the use of a strain of *Bacillus simplex* Daines (65) found that *T lignorum* produces a diffusible substance which is toxic to *A scabies* in an artificial liquid medium However, the toxic principle was rapidly destroyed by soil aeration It was believed doubtful that this fungus could be of much assistance in combating potato scab

Fellows (103) obtained field control of the take-all (*O graminis*) disease of wheat in Kansas by the application of certain organic materials such as chicken and horse manure, alfalfa stems and leaves, boiled oats and barley, and potato flour Garrett (117, 118) submitted evidence to prove that the factor chiefly controlling the subterranean spread of the pathogenic fungus along the roots of the wheat plant was the accumulation of carbon dioxide, with corresponding lowering of the oxygen tension, in the microclimate of the root zone This could best be maintained by periodical additions of organic manures Since organic matter low in nitrogen was more effective than high nitrogenous materials, it was postulated that the soil microflora uses the mycelium of *Ophiobolus* as a source of nitrogen The addition of nitrogenous materials, either in an organic or in an inorganic form, was believed to protect the mycelium of the parasite by offering a more readily available source of nitrogen Tyner (333) suggested that the differences in the microflora associated with the decomposition of different composts are largely responsible for differences in persistence and virulence of pathogens causing root-rots of cereals

Considerable reduction in slime-disease of tomato plants was effected by the addition of green manures to soil before planting (338) Organic materials high in nitrogen, as well as supplementary nitrogen sufficient for complete decomposition of the organic material, were found to be most effective Thom and Morrow (327) found that organic matter is most effective in depressing pathogenic fungi, during the period of its active decomposition King and associates (178) utilized the antagonistic action of soil microörganisms for the inactivation of pathogenic fungi in the soil before the crop-growing season Organic manures were added to the soil, in order to control *Phymatotrichum omnivorum*, the root-rot of irrigated cotton under continuous cultivation in Arizona By the use of the Cholodny slide technique, it was possible to demonstrate (84) that microbiological antagonism represents the true mechanism of the control process The development of saprophytic organisms was most profuse in the slides buried in the manured plots, whereas the mycelium of *P*

omnivorum was most abundant on the slides in the unmanured plots. It was suggested that parasitism of the fungal strands by bacteria is one of the reasons for the decline of the pathogen in manured soils. Henry (149) believed that the biological control by the soil microflora could even be directed against internal seed infection, since appreciable infection of surface-sterilized flaxseed was found to occur in sterilized but not in unsterilized soil.

The addition of bacteria to unsterilized soil exhausted by growing flax was found to lower the percentage of plants diseased by *Fusarium lini*. Novogrudsky (240) suggested the term "bacterization" for the process of treatment of seed with active bacteria in order to protect the plant against pathogenic fungi. It is concluded that the effect of bacteria on germinating seeds is due to the liberated bacterial products capable of depressing the development of parasitic fungi (15, 312, 184). Although, not in all cases conclusive, the results fully justify the hope that a better knowledge of the soil antagonists may lead, if not to complete control, at least to a certain amount of control over the numerous plant diseases caused by pathogenic fungi, especially those that persist for a certain length of time in the soil.

ANTAGONISTIC ACTION OF ANIMAL FORMS

Based upon the known fact that protozoa are able to feed upon bacteria, a theory was propounded, namely the "protozoan theory of soil fertility," that protozoa are responsible for the limited fertility of certain soils. According to this theory, the bacteria are viewed (291), as the sole agents responsible for the liberation of nutrients in the decomposition of soil organic matter and their transformation into forms available to higher plants. The protozoa, through their capacity of consuming bacteria, are considered as the agents controlling soil fertility. The increased fertility resulting from treatment of soil with heat and certain chemicals is regarded as a result of the destruction of the protozoa, the "natural enemies of the bacteria."

Subsequent investigations have not supported this theory (347). When protozoa were added to cultures of bacteria concerned in certain specific processes (53), they fed upon the bacteria and

brought about considerable reduction in bacterial numbers, this was not accompanied, however, by a detrimental effect upon the specific bacterial processes Pure cultures of bacteria were found to multiply in a nutrient medium until a limiting population was reached (43) Protozoa grew in that medium without bacteria only when the concentration of the food supply was increased 100 to 1,000 times, in the presence of bacteria, they grew also in the dilute solution The bacteria thus acted as collectors or concentrators of the food for the protozoa Bacterial numbers were thereby reduced, but bacterial activities continued The protozoa kept the bacteria below the saturation point, thus providing conditions for more continuous bacterial multiplication and for more complete oxidation of the organic matter

The effect of protozoa upon bacteria may thus actually be beneficial to natural processes (62, 214, 233, 322), as for example in the fixation of atmospheric nitrogen, the liberation of ammonia from proteins, and the formation of carbon dioxide from carbohydrates Cutler and Crump (62) suggest, therefore, that the presence of protozoa in the soil may keep the bacteria at a level of maximum efficiency The favorable effect of partial sterilization of soil may be explained (193) by the destruction of the bacterial antagonists

Various bacteria, especially *Pseudomonas aeruginosa*, have been found (52) to exert a toxic effect upon protozoa, limiting their development or bringing about their destruction (201, 257) The protozoa may develop a certain resistance against specific bacterial products (257) Protozoa are also known to feed on bacteria pathogenic to man (164, 102, 321, 267), animals, and plants (278, 154)

The importance of protozoa in the cycle of natural processes thus consists not in the mere destruction of bacteria, beneficial or injurious, but in establishing a variety of relationships with the bacteria, favoring the activities of some and impairing the activities of others

A variety of bacteria, fungi and nematodes have been found capable of destroying the larvae of various beetles in the soil Some of these organisms have thus been utilized for combating insect diseases of plants (216) Once these organisms have become

established in the soil, the beetles tend to disappear (123, 83) The ability of saprophytic nematodes to destroy root-knot nematodes has also been utilized for the destruction of the latter Heavy applications of organic materials to the soil were found (196) to result in a greatly increased population of saprophytic nematodes The decomposition of the organic residues supports large populations of plant and animal microbes destructive to the parasitic nematodes Here belong the nema-capturing fungi (74, 68), the non-trapping fungal parasites, predacious mites, as well as a variety of bacteria

CHEMICAL NATURE OF THE ANTAGONISTIC SUBSTANCES PRODUCED BY MICROORGANISMS

All bacteria were once said (26) to produce, at a certain stage of their development, antagonistic substances that are thermolabile and soluble in ether, alcohol and other solvents It is now definitely recognized that only certain species and frequently only specific strains of microörganisms are able to produce bacteriostatic and bactericidal substances These vary greatly in their chemical properties, in toxicity to animals, and in the mechanism of their action The differences are often more of degree than of kind For purposes of classification of these active substances the following criteria may be used (*a*) solubility in various reagents, (*b*) specific chemical nature, (*c*) specific bacteriostatic and bactericidal properties, (*d*) toxicity to animals and action *in vivo,* (*e*) nature of the organism producing such substances

Some are soluble in water, but not in organic solvents, some are soluble in alcohol or in chloroform, but not in ether and in acetone, some are soluble in these solvents, but not in water Chemically, these substances may be classified as, (*a*) lipoids and lipoid-like bodies, (*b*) pigments, (*c*) polypeptides, (*d*) sulfur-bearing compounds On the basis of their biological activity, they vary considerably, some acting in low and others in high concentrations, some act best upon gram-positive bacteria and less well upon gram-negative organisms, whereas others act chiefly upon fungi, some are primarily bacteriostatic, some are bactericidal, and some are bacteriolytic On the basis of their toxicity, they can be

classed as, (*a*) non-toxic or of low toxicity, (*b*) fairly toxic or (*c*) highly toxic Some of the substances have been crystallized, information has been gained concerning the proximate chemical composition of others, the nature of most others is still imperfectly understood (table 5)

The active bactericidal agents produced by *Pseudomonas aeruginosa* have received the most attention (93) Recent evidence points to *pyocyanase* being a lipoid and containing unsaturated fatty acids (150, 20) *Pyocyanin* is a chloroform-soluble pigment which can be synthesized The solution, left after the removal of the blue chloroform extract, when treated with ether gives a yellow pigment, a derivative of pyocyanin called (371) *hemipyocyanin*, which is also active (183) The *fluorescin* remaining in the culture, after the ether and chloroform extractions, is inactive Pyocyanin diffuses more readily than pyocyanase (259)

Pyocyanase acts upon a variety of bacteria, including *E coli* and *E typhosa*, but not *Proteus vulgaris* According to Gundel and Wagner (145), however, pyocyanase does not act upon the colon-typhoid group of bacteria Different strains of *P aeruginosa* may contain either pyocyanase or pyocyanin or both Pyocyanase was at first looked upon as an enzyme belonging to the class of nucleases (92) It had, even in very low concentrations, a marked destructive effect upon diphtheria, cholera, typhoid and plague organisms, as well as on pyogenic streptococci and staphylococci, the cholera cells were rapidly dissolved Bacterial toxins were rendered inactive in a few seconds The action of the preparation was found to be proportional to time and concentration, and inversely proportional to the numbers of bacteria acted upon It could be heated without much destruction, for two hours, in flowing steam, although some claimed (324) that the activity was thereby reduced The enzyme nature of pyocyanase gained further support because (1, 218, 358) the activity of certain bacterial enzymes heated at 100°, for 15 to 30 minutes, is not always destroyed

The enzymatic nature of pyocyanase was not universally accepted, however, because of its thermostability (179, 69) Dietrich (69) ascribed the action of pyocyanase to a change in osmotic

TABLE 5

Summary of certain selective bacteriostatic and bactericidal substances of microbial origin

SUBSTANCE	ORGANISM	PROPERTIES	REFERENCE
Actinomycetin	*Actinomyces albus*	Water-soluble, precipitated by alcohol, thermolabile, protein-like Lysis of dead bacteria	134, 359, 360
Actinomycetin (Lysozyme)	*A violaceus*	Water-soluble, ether- and alcohol-insoluble, thermostable, similar in action to lysozyme	185, 186
Actinomycin A	*A antibioticus*	Soluble in ether, alcohol, benzol and chloroform, not in petrol ether, orange-colored pigment, toxic, highly selective bacteriostatic action	348
Actinomycin B	*A antibioticus*	Soluble in ether, petrol ether, alcohol, but not in water, colorless, largely bactericidal	348
Gliotoxin ($C_{14}H_{16}N_2S_2O_4$)	*Trichoderma* sp, *Gliocladium* sp	Soluble in chloroform, benzol, alcohol, sparingly soluble in water, fungicidal and bactericidal	356
Gramicidin ($C_{74}H_{106}N_{14}O_{14}$)	*Bacillus brevis*	Soluble in ether, acetone, lytic against gram-positive bacteria, toxic, active *in vivo*, polypeptide	77, 162
Penicillin	*Penicillium notatum*	Alcohol-soluble, thermolabile, low toxicity	104, 272, 58
Pyocyanase	*Pseudomonas aeruginosa*	Lytic on many bacteria, thermostable lipoidal, activity largely due to unsaturated fatty acids	92, 18, 94, 24, 100, 183, 259, 348
Pyocyanin	*P aeruginosa*	Chloroform-soluble, blue pigment, thermostable, bactericidal action limited	150, 143, 183, 371
Tyrocidin ($C_{44}H_{60}N_9O_{11}$)	*Bacillus brevis*	Insoluble in ether, soluble in absolute alcohol, lytic to gram-positive as well as gram-negative bacteria, toxic, polypeptide	77, 162

pressure. Raubitschek and Russ (269) emphasized that the solubility of the substance in ether, chloroform and benzol does not speak for its being an enzyme, nor the fact that temperatures of 0° to 37° do not influence its activity It seemed to be merely a lipoid (145, 247, 344, 150, 75, 20) Hettche (150) reported that pyocyanase consists of a phosphatide, a neutral fat and a free fatty acid The bactericidal properties were said to be due to the free fatty acids A definite relation was found between the number of double bonds in pyocyanase and its bactericidal properties (150, 20) This information may be correlated with the finding (75) that most fatty acids exert bactericidal and bacteriolytic effects upon gram-positive bacteria, whereas gram-negative organisms are not lysed, which thus tends to explain the greater effectiveness of pyocyanase upon the former

The pigment also was found (143) to possess lytic properties, a 1 1000 dilution of pyocyanin being able to lyse *E coli* in 6 hours Kramer (183) believed that pyocyanin acts only on gram-positive bacteria Pyocyanin was said to be most effective in young cultures and pyocyanase in old cultures (150) The pigment appears to influence bacterial respiration (87)

An enzyme capable of decomposing the capsular substance of pneumococci has been isolated by Dubos and Avery (78, 76) from certain soil bacteria This enzyme is highly specific, acting only upon one type of pneumococcus which is thereby rendered susceptible to destruction by phagocytosis It is produced only in the presence of the capsular polysaccharide or of the aldobionic acid derived therefrom The enzyme seems to be associated with a protein which passes through a collodion membrane with an average pore size of 10 6 μ, but is held back by pores of 8 2 μ (78, 127, 305)

The credit for having first isolated, from spore-forming bacteria, specific chemical compounds in crystalline form, aside from the work on the bacterial lipoids, is due to Dubos (77) By applying the principle of enrichment of soil with pathogenic bacteria, he isolated a spore-forming bacterium capable of bringing about lysis of various living gram-positive organisms The active material, *tyrothricin*, is protein-free, non-volatile, non-dialyzable through

collodion, and heat-labile It is alcohol-soluble, water-insoluble, stable at alkaline reactions, but is rapidly inactivated at reactions more acid than pH 5 5, even at room temperature When redissolved in a neutral medium, the substance exhibits the lytic activity of the original solution from which it was extracted

The bactericidal material can be further purified and separated into several crystalline preparations *Gramicidin* is obtained by extracting the crude alcohol-soluble material with a mixture of acetone and ether, evaporating, dissolving in boiling acetone and cooling The crystals are spear-shaped, colorless platelets, melting at 228-230° The substance yields 62 7 per cent C, 7 5 per cent H and 13 9 per cent N, with a molecular weight of about 1400, suggesting the empirical formula $C_{74}H_{106}N_{14}O_{14}$ It has no free basic nor acidic groups, and contains 10 molecules of α-amino acids of which 2 or 3 are tryptophane residues

A second fraction, designated *tyrocidin*, is insoluble in acetone or ether, and soluble in acidified boiling absolute alcohol, from which it precipitates on cooling The product is recrystallized from acidified absolute methanol yielding clusters of microscopic needles melting at 237-239°, with decomposition It is similarly built up of amino acids but contains one free carboxyl, one tryptophane and one tyrosine group per molecule The molecular weight is given as about 900 and the compound represented by the formula $C_{44}H_{63}N_9O_{11}$ Gramicidin acts against gram-positive bacteria, while tyrocidin is also active against gram-negative bacteria (162) The two substances are highly toxic, especially gramicidin of which 0 3 mg kills a mouse when injected intraperitoneally (203) These or similar compounds appear to be widely distributed among spore-forming soil bacteria (157, 320)

Actinomycetes are known to produce at least three types of active substances (*a*) *actinomycetin*, (*b*) *lysozyme*, and (*c*) *actinomycin* The first is water-soluble (134, 359, 360) and acts primarily against dead bacteria, although living bacteria were later also found (131) to be affected It is thermolabile and destroyed by ultraviolet rays and by strong acids, but not by mild antiseptics It is precipitated by acetone, alcohol and ammonium sulfate Borodulina (30), however, found that the toxic agent of actino-

mycetes antagonistic to *Bacillus mycoides* is thermostable These differences in heat resistance point to possible chemical differences in the nature of the preparations produced by different actinomycetes Other Russian investigators (185, 186) also found a type of actinomycetin which is filterable and resistant toward radiant energy, it is said to resemble egg-white lysozyme, but not to be identical with it It is soluble in water, but insoluble in ether, petroleum ether, benzol and chloroform

Actinomycin obtained from *A antibioticus* (348) is separable into two fractions A, soluble in ether and in alcohol, but not in petroleum ether, and giving a clear solution in water, B, soluble in ether and in petroleum ether, soluble with difficulty in alcohol, and giving a turbid suspension in water *Actinomycin A* is bright red in color, giving a yellow solution even in concentrations of $1\mu g$ per ml, it possesses extremely high bacteriostatic properties, but is rather slowly bactericidal It is produced in liquid and in solid, inorganic and organic media, and is completely removed by charcoal, it is not affected by heat, and is only partly removed by passage through a Seitz filter *Actinomycin B* has little bacteriostatic action but is actively bactericidal

Penicillin, produced by *Penicillium notatum*, has a strong antibacterial action (104) Gram-negative bacteria are least sensitive and pyogenic cocci most susceptible It is soluble in alcohol, but not in ether or chloroform, it is inactivated by oxidation and by evaporation at 40 to 45°, in acid and alkaline solutions, although it is fairly stable at pH 5 to 6 However, at pH 2 0 it is completely soluble in ether The substance is extremely labile (58) It does not dialyze through collodion membranes and resists heating at 60 to 90° for short periods, and 100° for 5 minutes, but not for 10 minutes (272) Light (rich in ultraviolet rays), as well as oxygen, hydrogen and carbon dioxide bubbled through the medium prevent its formation or cause its destruction The most active preparation completely inhibits the growth of staphylococci in dilutions of 1 800 If permanently adjusted to pH 6 8, penicillin retains its potency for 3 months

Another type of compound produced by certain fungi, belonging to the genera *Trichoderma* and *Gliocladium*, was isolated by

Weindling and designated as *gliotoxin* (356) The period of greatest activity was produced in 2 days, soon after germination of the fungus spores The nature of the medium and the final reaction are important for the production of the active substance It is extracted from the culture with chloroform, the latter is then distilled off and the residue taken up in a small amount of hot benzene, or 95 per cent alcohol, from which, on cooling, silky white needles crystallize out The substance, recrystallized from benzene or alcohol, has a molecular weight of 347 and the chemical formula $C_{14}H_{16}N_2S_2O_4$ It inhibits the growth of *Rhizoctonia* hyphae up to a dilution of one in three millions The crystals, as well as the crude material, are also toxic to *Trichoderma*, but the minimum lethal dose is about 40 times greater than that for *Rhizoctonia* The active substance is sparingly soluble in water

DISEASE CONTROL BY UTILIZATION OF ANTAGONISTIC MICROORGANISMS

Numerous attempts have been made to utilize microörganisms for the control of various diseases in man, animals and plants As early as 1877, Pasteur (251) noted that the development of anthrax in sensitive animals can be repressed by the simultaneous inoculation of *Bacillus anthracis* with various other bacteria Pasteur may thus be looked upon as the first one to advance the idea of bacteriotherapy

In 1885, Cantani (44) treated a patient suffering from tuberculosis with a culture of a saprophytic organism, designated as *Bact termo* The effects were highly favorable He expressed the hope that other infectious diseases of a local nature or readily accessible might in time be treated with saprophytic bacteria that are antagonistic to the pathogens

Emmerich (91) found that anthrax can be controlled by the simultaneous inoculation with other bacteria, such as species of *Streptococcus*, organisms once looked upon as agents for rendering the organism resistant to all bacterial infections Pawlowsky (252) obtained resistance against anthrax infection by inoculation with Friedlander's bacillus Bouchard (31) had good results from simultaneous inoculation with *Pseudomonas aeruginosa*, however,

this did not impart permanent resistance to the animals There are numerous other instances on record of reduction in pathogenicity of one organism by the presence of others (23, 50, 94)

In order to overcome the destruction of pyocyanase in animal tissues, Emmerich and Löw prepared "immunoproteids," which consisted of a mixture of pyocyanase with blood or other animal tissues Rabbits could thus be protected against anthrax Woodhead and Wood (369) used a sterilized ten-day-old culture of *P aeruginosa* and obtained healing action against anthrax infection, or at least a delay in the course of its development Vaerst (339) succeeded in curing rabbits infected with anthrax by means of a pyocyanase preparation (11), and pyocyanase was soon (94) applied against various infections

There has been considerable disagreement over the therapeutic action of pyocyanase This was largely due to the variation in the nature of the preparations (200), and especially, the strain employed Kramer (183) showed that the activity of pyocyanase depends on such factors as the nature of the strain, since not all strains are equally effective, the composition of the medium, glycerol-containing media being most favorable, and the method of extraction of the active substance

Gaté and Papacostas (120) observed that mixed infections were usually mild (49), mixed cultures of the bacillus of Friedländer and *Corynebacterium diphtheriae* gradually gave a predominance of the former on repeated transfer No toxin was produced when the filtrate of the culture of the antagonist was used for growing the diphtheria organism The therapeutic use of filtrates was, therefore, suggested The subcutaneous injection of beer yeast was found (332) to protect rabbits against fatal streptococcus and staphylococcus infections

Gratia (131) prepared a vaccine for immunization purposes by allowing the specific organism to be acted upon by an antagonist The culture was heated to 56°, dissolved by the use of an actinomyces, and the resulting solution was employed as the vaccine Bumm (42) used a preparation designated as *neocolysin*, made up of living, proteolytic bacteria, which gave good results in chronic purulent conditions such as osteomyelitis The bacteria were

supposed to continue growing as long as there was dead tissue available The application of bacteriotherapy for treating chronic infections of the middle ear (266) and of actinomycosis (71) has also been suggested

Besredka (17) used a filtrate from an anthrax culture for dressings or for intracutaneous injections, the results obtained were as good or even better than those obtained with the bacterial vaccine Later, he utilized staphylococci and streptococci for similar purposes He believed that a substance (*"antivirus"*) secreted by the bacteria is dissolved in the filtrates, which substance checks further growth of the bacteria Although in many studies of this phenomenon (47, 209, 243), suggestions were made that the favorable effect is due entirely to the medium (4), the therapeutic results of Besredka have largely been confirmed The general opinion is that the filtrate does not act upon the infecting bacteria directly, but rather upon the tissue by way of local immunization Although an occasional increase of resistance caused by the non-specific filtrates has been found, the protection produced by specific filtrates seems to be more intense and more dependable (243)

Morgan and Harvey (222) showed in 1909 that *E typhosa* is inhibited by the free growth of antagonistic bacteria As a result, there was believed to be particular danger when pasteurized milk becomes contaminated with this pathogen Metchnikoff (217) suggested utilization of the antagonism between lactic acid and proteolytic bacteria for repressing the growth of the latter Pure cultures of lactic acid bacteria are introduced into the food of man in order to repress in the intestinal canal the proteolytic bacteria which are supposed to bring about the intoxication of the system In recent years, *Lactobacillus acidophilus*, an inhabitant of the human intestine and possessing antagonistic properties against undesirable intestinal bacteria, has come to the front

Fleming (104, 105) suggested that penicillin could be used as a dressing for septic wounds This preparation has little toxic effect and seems to be superior to dressings containing active chemicals The difficulty in the use of penicillin, as in the case of pyocyanase, is due largely to the fact that the preparation does

not maintain its potency for more than a few weeks Penicillin is not related to any chemotherapeutic substance now in use (48) As compared with sulphonamide drugs, it is (2) not inhibited by tissue constituents and pus, thus offering a definite advantage from a chemotherapeutic point of view

Gramicidin injected intraperitoneally into white mice, was found (77) to exert a therapeutic action against experimental peritonitis caused by pneumococci and streptococci However, it is almost completely ineffective when administered by the intravenous, intramuscular, or subcutaneous route Particularly favorable results were obtained (198) with chronic mastitis Sterile mineral oil was found to be a suitable, non-irritating medium for its administration Of 31 quarters of cows naturally infected with *Streptococcus agalactiae* and treated by the gramicidin-oil mixture, 26 seemed to have responded by a complete disappearance of the streptococci The infection in some of the cured cases was of a severe chronic nature Gramicidin-like preparations have also been used (212) in the treatment of local infections, such as osteomyelitis

Thus far, the utilization of specific microbial products for the control of plant diseases has made comparatively little progress (191, 60, 241) Various Russian investigators (15, 56, 240, 234) recommend the inoculation of plant seeds with bacteria ("bacterization" of seed) in order to combat infectious diseases This phenomenon appears to involve, however, various complex soil-plant-microbe interrelationships (181, 316)

RETROSPECT

Ranging between the phenomena of true parasitism, where one organism lives in or upon the living body of another, and true saprophytism, where one organism merely destroys the waste products and dead cells of the other, there is a wide range of relationships between living systems which may be designated as associative and antagonistic in the first, one organism assists the other, whereas in the second, one organism is injurious to the other The antagonistic effects vary from those of space relations between the antagonist and its neighbor, where the prox-

imity of one organism is injurious to the growth of the other, to the production by one organism of definite chemical substances which injure or interfere with the growth of the other As is generally the case with parasitic relations, the antagonized organism frequently develops a protective mechanism against the antagonist and is often able not only to neutralize its effect but even to destroy it Often a balanced condition is established between the two organisms, where both are able to survive the antagonistic effects

Numerous instances are found in nature where the presence or introduction of one organism leads to the destruction of another, whenever the latter, in its turn, leads a parasitic existence upon higher forms of life, the antagonist becomes a beneficial agent to these in their efforts to overcome the effects of the parasite or to destroy it This phenomenon is largely influenced by the host species, by the type of parasite, as well as by the nature and degree of infection

Just as the pathogenic bacteria are commonly believed to have evolved from the harmless saprophytes, so the organisms possessing antagonistic properties must have evolved in some manner from those that do not possess such properties Otherwise, how could one explain the capacity of certain strains of such common universal saprophytes as *Bacillus subtilis* and *B mycoides* to produce powerful agents capable of bringing about the lysis of numerous pathogenic and saprophytic bacteria? The fact that many pathogenic bacteria produce substances antagonistic to their own kind or to other pathogens has actually been utilized in an attempt to combat these agents of infection

The mechanism of the action of the antagonist varies greatly and appears to depend largely upon the specific nature of the active substances Some of these appear to be produced by only one organism, others are produced by many organisms, some antagonists, on the other hand, produce more than one active substance The action of the antagonist may be either primarily bacteriostatic or largely bactericidal, the latter may or may not be accompanied by the lysis of the antagonized cells Whereas in many cases the living cells of the prey are lysed by the antagonist, in other cases, the dead cells are lysed more readily

The substances produced by these antagonists also vary greatly in their effectiveness, when injected into the animal body. Some possess a low toxicity (pyocyanase, penicillin), others are highly toxic (gramicidin, and especially actinomycin). This as well as differences in the solubility of these preparations account for the differences in their utility. Some appear to act better when applied to combat local infections, whereas others may become useful in attacking more generalized infections.

There is increasing appreciation of the fact that nature harbors many unknown organisms that are capable of combating disease-producing bacteria, fungi, worms and insects. Our knowledge of the activities, potentialities, and importance of these microbes is still incomplete. Man, in his struggle for existence succeeded, before the development of microbiology, in domesticating and utilizing the activities of many microbes. Here belong the lactic acid bacteria of milk, the wine-fermenting, beer-fermenting and bread-fermenting yeasts, the silage-producing, sewage-digesting, compost-producing and soil-inhabiting microörganisms. However, these represent only a small fraction of the microbial world. It is possible that we are finally approaching a new field of domestication of microörganisms for combating the microbial enemies of man and of his domesticated plants and animals.

Many practices in surgery, as well as old-time remedies, are based upon the creation of conditions favorable to the development of antagonistic microörganisms. The method of cast surgery, introduced in the Spanish Civil War, and the application of urine to cracked skin and local wounds, as practiced by certain farmers in this country may serve as illustrations. The chance contaminants may possibly serve as the antagonistic agents; to what extent the application of pure cultures of antagonists may improve such practices still remains to be determined. Trueta (331) states that plaster-treated wounds which gave, without the use of antiseptics, such marvelous effects during the Spanish Civil War, were found to contain aerobic bacteria with no one group predominating, except for *Bacterium pyocyaneum* tending to become more numerous, when the healing process has been established.

The utilization of fungi and bacteria for combating plant

diseases has also been variously attempted The difficulty here is to establish the antagonist in the soil This can be done only when conditions are modified, as by addition of stable manure or other plant and animal residues, which favor the development of the antagonists Among the various other possibilities for utilizing antagonistic microörganisms in combating disease-producing and other injurious organisms, the methods of control of insects and other lower animal forms occupy an important place The Japanese and other Asiatic beetles, which are so highly destructive to plants, have thus been combated rather successfully

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THE WASSERMANN ANTIGEN AND RELATED "ALCOHOL-SOLUBLE" ANTIGENS

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The attempt is made in this review to present material scattered rather widely in scientific periodicals—some of them not everywhere equally obtainable—and considered in textbooks and handbooks mostly from the viewpoint of its possible bearing on the sero-diagnosis of syphilis This is explained by the circumstance that substances of the type under consideration originally came into the orbit of investigation as the antigen of the Wassermann reaction and allied methods for the diagnosis of syphilis Subsequent immunological investigation has revealed the rather ubiquitous presence of such substances in living matter Little, however, is known about their biological significance It will be seen that during the past fifteen years there has accumulated a considerable amount of data which, though often loosely interconnected and sometimes awkward in chemical technic, is indicative of future developments of considerable interest Thus, this survey is undertaken not so much from a wish of enumerating achievements as from the desire to present a basis for future investigation

This review attempts to cover the work done since 1925 Older results are mentioned only insofar as they are thought necessary for perspective to the newer information For older references see (1) and (2) The very numerous papers on antigens of the Forssman group are also mentioned only insofar as they are pertinent to the present problem

In this discussion we shall avoid the collective name under which these antigens are commonly grouped, namely the term "lipid" The only thing that substances comprised under this name have in common is the readiness with which they can be extracted by organic solvents from tissue or other organized matter In this category are substances as heterogeneous as

sterols, lecithin, cephalin, cerebrosides and the complicated phosphatides of bacteria, and it is obvious that the solubilities of some of these substances in the pure state may be of an entirely different order Thus we think it better to forego the convenience of the term lipid in order to avoid another contribution to the lack of discrimination which this expression has furthered in the past We prefer to use the purely descriptive, provisional term "Wassermann-type substances" It will be shown that this expression excludes the well-known major constituents present in alcoholic extracts of organized matter, such as cholesterol, phosphatides and cephalin

Origin of present conception of the nature of the Wassermann reaction The Wassermann reaction was originally thought to be an application of the complement-fixation method, (which was quite new at that time) to the problem of sero-diagnosis of syphilis, using as an antigen the livers of syphilitic fetuses because they contained an abundance of spirochetes (3) Two observations were soon made which shook the belief that the Wassermann reaction was directed specifically against *Treponema pallidum* it was demonstrated that alcoholic extracts were as good or better antigens than aqueous extracts (4), second, it was found that alcoholic extracts from normal organs served just as well (4 to 6) The attempts to arrive at an understanding of this situation prior to the year 1925 will not be considered here If the Wassermann reaction were a genuine antibody-antigen reaction at all, the observation could seemingly be explained only by envisioning an antigen of non-protein nature and derived from the host itself, thus apparently violating two principles generally accepted at the time, namely that of protein alone being antigenic and that of the "*horror autotoxicus*" The hypothesis offered by E Weil and H Braun (7) contained therefore two heretical conceptions, namely that of a "lipoid antigen" derived from tissue of the host, and of an "autoantibody" directed against it[1] A further pre-

[1] An immune precipitation with material derived from the host's body was first described by Centanni (see 7), who found in the blood of sheep infected with distomum an antibody precipitating sheep liver extract The development of the concept of autoantibodies cannot be discussed here

requisite for this hypothesis is the acceptance of a special ability of *Treponema pallidum* to function as an activator of the tissue antigen There is no direct evidence available on this matter, but differences found in the ability of "conveying" (which will be discussed in detail later on) make a conception of this kind plausible

In 1924, Landsteiner (8, see also 9) demonstrated that specific antibody formation of the Forssman type could be evoked if alcoholic extracts containing this antigen (for instance from horse kidney) were injected in admixture with a protein (hog serum) The term hapten (10) was introduced to designate the peculiar immunological behavior of substances of this type, which react well *in vitro* but evoke antibody response only when introduced in combination with a protein

IMMUNOLOGICAL BEHAVIOR

Experimental antibody production The resemblance (11) of the immunological behavior of the Forssman antigen to that of the antigen contained in alcoholic organ-extracts (which react with syphilitic serum) led logically to experiments with alcoholic extracts from rabbit tissue plus hog serum (12 to 15) Abundant antibody response in rabbits was found, and the antibodies were recognized as corresponding in all essential aspects to those observed in the serum of syphilitic man and in the serum of animals infected experimentally with syphilis Both complement-fixation and all types of flocculation reaction with alcoholic extracts were obtained

The difference of these antibodies from those produced concomitantly against the protein conveyor is clearly defined (13) and can be confirmed by absorption tests (16) A much longer period of immunization is needed for obtaining this antibody response than is necessary for evoking antibody to the protein (13)

Conveyor There is no indication of what actually happens in a mixture of a "lipid" hapten and serum in order to effectuate the antigenicity of the former Landsteiner (8) thought that

the hapten might enter some kind of a loose combination with the conveying protein Another suggestion was (12, 13) that the protein did not enter into any true chemical action with the hapten, but served only as a kind of envelope which would enable the non-protein matter to enter the cells This was expressed under the name "Schlepper Funktion," a term which is not easily translated The best translation seems to be conveying function, and thus what is called in German "Schlepper" will here be termed conveyor

A third alternative explanation of the conveying function was suggested by the observation that in some cases the protein conveyor could be replaced by adsorbents like kaolin or $Al(OH)_3$ This was reported (17 to 19) to be the case for the Forssman antigen, and also for antigen contained in brain extracts However this observation cannot be generalized because other antigens have not been found to be "completed" by adsorbents (20, 21) Experimentation with adsorbed antigens is a difficult matter because suspensions of this type are often poorly tolerated when introduced parenterally (20, 22)

It would be interesting to follow up this matter more closely It is a situation quite peculiar for this kind of antigen, somewhat in between a full antigen and those true haptens which need actual complex-formation before being effective in provoking specific antibody formation in animals In the case of the Forssman antigen, it is known that the necessity for a conveyor is only a relative one insofar as occasional slight antibody formation may occur with the hapten alone There are several observations which might be interpreted as indicating a similar behavior of antigens of the Wassermann type (14)

Aqueous suspensions from syphilitic fetal liver cause the appearance of Wassermann antibodies when injected into rabbits, an effect not obtained with normal liver (23) (see below) The specific precipitate consisting of human syphilitic serum combined with antigen has been found to be an excellent immunizing agent (27 to 29) The same is true for the Forssman antigen-antibody complexes (28)

A very small amount of protein suffices for serving as a conveyor

(24) Sera of different species are effective, but they are not identical in their quality of conveying (25, 26) Horse serum is inferior to pig serum (25), human serum seems to be a good conveyor (16) Serum denatured by heating is decidedly inferior (27) Treatment of serum with diazotized atoxyl seems to destroy the conveyor function (30) Rabbit serum, either native or heated, is not a conveyor in rabbits (22, 34)

Tissue after alcoholic extraction is capable of acting as a conveyor for liver extract, but not for Forssman antigen (probably because of denaturation) (23) Bacteria are able to exercise a conveyor function (31, 32), and so does an alcoholic extract from bacteria (33)

Peptone, peptides (glycyl-lysyl-glycine) and polypeptides do not act as conveyors (35, and unpublished experiments of the author)

Lack of anaphylactic reaction In contrast to the development of antibodies highly reactive *in vitro*, neither active nor passive anaphylaxis is obtained against antigens of the Wassermann type (36 to 38) Reports to the contrary (39) are open to objection and could not be duplicated (15, 40) It is quite possible that future investigation with purified antigens will cause modification of this statement Floccules from syphilitic serum plus antigen do not sensitize guinea pigs to the extract, but they do sensitize to both normal and syphilitic human serum (41) (It should be noted that a hapten itself may be well qualified for causing anaphylaxis, for example, recall the reactions obtained with pneumococcal carbohydrates)

Recent reports (32, 42) concerning passive anaphylaxis against a thermostable antigen from brain "associated with myelin and probably derived from it," may require, if this antigen is indeed linked to an antigen of the Wassermann type, some qualification of the generalization just given

No experimental antibody production in man and animals other than the rabbit A number of attempts have been made to duplicate in other species the antibody formation obtained in rabbits The results have been unconvincing, and most of them entirely negative Experiments in this direction have included

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mice (43), rats (44, 45), guinea pigs (26, 27, 40, 46 to 48), and geese (49) Wassermann (50) did announce a positive result in goats, but a detailed report on this subject has never been made

This situation remains still to be explained It may be recalled, however, that observations not unlike this one have been recorded, for instance, the differences in antibody response in various species of animals toward pneumococcal carbohydrate In this connection, it may be recalled also that the rabbit is generally one of the most prolific formers of circulating antibodies

Rats and guinea pigs do not form antibody against *Trypanosoma brucei* under conditions where rabbits and humans do produce an antibody of the Wassermann type (51) Nor is antibody obtained in guinea pigs after treatment with alcoholic extract from typhoid bacilli, which does cause a response in rabbits (52, 53) A number of experiments on human beings have yielded unconvincing or negative results (54, 55) There is also no formation of the Wassermann-type antibody after (therapeutic) treatment with milk (55, 56)

It must be borne in mind that in most experiments of this kind the number of treatments was limited and that injection could not be made intravenously (57) This is important It has been found (14, 27) *that even in rabbits subcutaneous or intra-ab*dominal injections do not evoke an antibody response to the combination of alcoholic extract and pig serum (Intraperitoneal injection, however, has been reported to be effective with the aqueous brain-suspension mentioned below (58) and with syphilitic liver-suspensions (23) and pig heart (59))

In Vitro Reactions Rabbit serum is, for reasons unknown, liable to cause non-specific deviation of complement more than that of most other species Omission of the necessary safeguards against such occurrences has made valueless quite a number of experiments reported in the literature However, the complement-fixation method is entirely reliable if properly conducted (3, 13, 27)

Fortunately, one is not confined ordinarily to the use of the complement-fixation test, because flocculation tests are obtained easily in most, but not all, cases The flocculation tests are not

entirely free from the pitfalls of the complement-fixation test, but they are comparatively safer, especially under conditions where experience in the technique of complement-fixation is lacking In any case, one of the most essential precautions in any method is the use of dilutions well out of the danger zone, but this of course makes it difficult to obtain proper information in cases where antibody titers may be low

The range of sensitivity of complement-fixation tests and of various flocculation tests differs somewhat, and this is true not only for the lower limits of antibody titer but also for the higher ones, because inhibition by excess does occur (13, 60) It is highly desirable to use wherever possible both complement-fixation and flocculation tests together This desirability of using more than one technique is equally important for the diagnostic application in human syphilitic infection, a point which might be stressed once more because it is too often overlooked

The conditions for combination between antigens of the Wassermann type and the antibody have been studied quantitatively Relatively large amounts of antigen combine with relatively small amounts of antibody (61, 62) Investigation of the mechanism of flocculation shows that antibody can account for up to 20 per cent of the total precipitate, however, much smaller amounts are sufficient for precipitation (61 to 63) It is calculated that coverage of $\frac{1}{20}$ of the surface of the antigen particles (cholesterinized heart-extract) is enough to cause flocculation Precipitates in normal serum do not take up any protein from the serum The isoelectric point of the antigen-antibody complex lies between pH 4 1 and 4 8 or even higher (The isoelectric point of the antigen alone is at pH 1 8) The critical potential of the antigen is 1 to 5 mv, and that of the antibody-antigen complex, 10 to 15 mv (63)

A separation of antigen from antibody can be effected by moderate heat (64, 65) and in concentrated NaCl solution (66), (for an older procedure see (59)) These methods are used as a means of distinguishing between specific and non-specific reactions in the practice of syphilis diagnosis the specific floccules will

liberate antibody which can be demonstrated, while the non-specific floccules will obviously not deliver any antibody Forssman antibody has been found to be detachable from stromata by alkali or water (67)

With purified antigens, it can be shown that one microgram suffices for complement-fixation or flocculation (68) In the inhibition tests with purified Forssman antigen still smaller amounts are found to be effective (67) However, for the stimulation of antibody response, synthetic or highly purified antigens are, as a rule, less effective than are crude extracts (69)[2]

Antibodies of this type seem to be more thermolabile than antiprotein antibodies (71, 72)

Agents fortifying in vitro reactions One peculiarity of reactions of this type is the enhancement of the effect of the antigen *in vitro* by means of finely dispersed agents This observation, made more than thirty years ago and put to a practical application of considerable importance (see 1), is a purely empirical one, and it has contributed its part to the confusion concerning the nature of the test for syphilis Cholesterol was found to be especially convenient for this purpose and has maintained its position as a fortifying agent ever since It was demonstrated that its peculiar qualification is based on the formation of microscopic crystals when it is added to aqueous solutions These crystals furnish the surfaces on which the antigen proper is adsorbed and on which the antigen-antibody reaction takes place (61 to 63) It is, however, not yet known whether this adsorption contributes materially to the ease of combination or whether it is merely an adjuvant to aggregation and the formation of visible particles This latter action certainly plays an important role in flocculation, however, the effect is just as marked in complement-fixation, and there the problem of this effect remains shrouded in the veil of our lack of knowledge of the action and mechanism of complement-fixation generally Recent investigations suggest once more a definite optimal ratio of cholesterol (73)

[2] In the case of purified Forssman antigen large amounts (100 mgm per dose) were found to be less effective for eliciting antibody formation than were small doses (1 mgm) (70)

Cholesterol is by no means the only substance active in this way (74) Cholesterol derivatives are found to be quite similarly active if used in proper quantities (75) Commercial lecithin is another agent of this kind (76), (commercial lecithin preparations may, however, contain considerable amounts of cholesterol) Other examples of non-specific fortifying agents are cited in (77)

Special mention should be made in this connection of the effect of phenol (78, 79) It may be that this effect is similar to that of cholesterol, a physical one, effectuated by the creation of fine protein precipitates It is, however, possible that the effect is a chemical one in the sense that phenol may break up the combination between tissue protein and antigen There are no direct chemical observations on this point "Unspecific" reactions caused by phenol-alcohol have been suggested as due to the liberation of antigen from serum (80)

A similar mechanism may be responsible for the formation of antibody of the Wassermann type observed in the sera of rabbits immunized with diazotized atoxyl-protein or metanilic acid-protein (30)

OCCURRENCE AND BIOLOGICAL ASPECTS OF WASSERMANN-TYPE ANTIGENS

Ubiquity of the Wassermann antigen Materials reacting with syphilitic sera have been found to be present in practically all living matter, especially in organs of all kinds of animals (1, 81) Alcoholic extracts of microorganisms contain the active principle quite frequently (see table 1) and so do alcoholic extracts from plants (82, 83)

However, this agent is not the only alcohol-soluble antigen present in living matter The alcohol with which it is commonly extracted quite often extracts other substances of a more specific nature Thus it was found (84) that Forssman and Wassermann antigens are present together in alcoholic extracts of organs from animals of the heterophile group [3] The two antigens can be separated by specific absorption (47)

[3] For this reason, it is inadvisable to use tissue of animals belonging to the Forssman group for the preparation of extracts for the serodiagnosis of syphilis (85)

Antigens with organ-specificity Alcoholic extracts of rabbit hearts, livers, and kidneys were found to react preponderantly with sera obtained by the immunization with the respective organ extract (plus hog serum) and only to a lesser degree with extracts of different organs (13, 86, 87) This situation is more marked in the case of extracts from brain ("neurohapten") (26, 45, 88 to 90) Immunological differentiation of grey and white substance has been demonstrated (91) Brain specificity appears in the 3rd to 4th month of human embryonic development (91, 105) Antigens of considerable organ-specificity can be found in alcoholic extracts of blood cells, testis (92), ovary (93), thyroid (94), hypophysis (95), and so on (49, 96 to 101) Leucocytes also contain a highly specific antigen (103, 104) The degree of specificity varies not only from organ to organ, but also within each individual immune serum Cross reactions are frequent, and they indicate relations that can be more or less clearly demonstrated by absorption tests A specially pointed example of this is the cross reaction between brain and testis (92, 102) which appear to have in common an antigenically rather active material, the same antigen has been recently found to be present in alcoholic extracts of corpus luteum (but not in other ovarian tissue) (225) It would be of interest to know whether the antigen common to brain, testis, and corpus luteum is identical with that causing the cross reaction between brain extracts and spirochetes (100)

Both older and more recent findings (42, 58, 88, 89) suggest that an organ-specific antigen may be obtained from brain by autolysis or by the action of microorganisms It is described as strictly tissue-specific and not species-specific, heat stable, non-dialyzable, and linked to myelin for chemical reasons as well as from the fact that it is not found in fetal brain, more recently, however, it has been suggested that it may be rather of protein nature (32) It is unknown at present how this water-soluble antigen is related to the material contained in alcoholic extracts[3a]

[3a] In a paper which came out when this review was already in press, the existence of two different heat-stable antigens in brain tissue has been demonstrated One of them is soluble in alcohol and, presumably, is the one which is active in

This antigen, associated with the white substance of the brain, is also present in the nerves It presents the same antigenic relationship to testis as was pointed out for alcoholic brain-extract, and thus it is conceivable that the water-soluble antigen may be the bearer of this relationship

Injections of milk (55, 116) and of yolk (117) have been found to cause the formation of antibodies of the Wassermann type in rabbits, in these cases no conveyor is necessary

Antigen with tumor-specificity Malignant tumors contain more or less regularly an agent which goes over into alcoholic extracts and which is common to most kinds of malignant growth (106 to 111) An antibody against this tumor-specific agent is found not only in the serum of animals immunized with tumor extracts, but also in a considerable percentage of human beings affected with malignant growth (112) It remains to be seen whether this tumor-specific antigen from alcoholic extracts is identical with that found in "heavy material" sedimented by centrifugation at 27,000 rpm from aqueous extracts together with Wassermann, organ-specific and Forssman antigens (222) "However as matters stand now this antibody is not found frequently enough, and especially not early enough in the course of the disease in order to be of diagnostic importance" (113, see also 114) There is also found a rather high percentage of "unspecific" reactions (114, 115), explained as "Zerfallsspecifitat" (106)

Antigens from animal parasites and bacteria Specific alcohol-soluble antigens have been obtained from tapeworms (118, 119), hydatid fluid (120) and bilharzia (121) In bacterial extracts the situation can be highly complicated Specific antigens, antigens common to large groups of bacteria, and antigens of the Wassermann or the Forssman type have been found, and the various antigens are often present together in the same extract This situation is presented in table 1, which lists the antigens which have been found in alcoholic extracts, either alone or in company The blank spaces mean that no data have been re-

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alcoholic brain extracts The alcohol-soluble and the alcohol-insoluble antigens could be differentiated by immunological methods Henle, W, Chambers, L A, and Groupe, V 1941 J Exptl Med, 74, 495

ported All of the reports refer to the antigen response in rabbits The injection of alcoholic extracts from typhoid bacilli into guinea pigs apparently does not lead to antibody formation (52, 53)

Antigens from spirochetes and trypanosomes Of special interest are two types of microörganisms, namely *Treponema pallidum* and the trypanosomes (51, 134, 135) The behavior of the former will be discussed in detail later on Trypanosomes are

TABLE 1

Antigens to be found in alcoholic extracts of bacteria

ALCOHOLIC EXTRACTS FROM	HOMOLOGOUS ANTIBODY FORMATION IN RABBITS	CROSS REACTIONS			REFERENCE
		Bacterial	"Wassermann" (tissue extract)	Forssman	
Proteus X_{19}	+		+	+	33, 122, 123
Typhoid bacilli	+				122, 124
Paratyphoid A and B bacilli	+				122, 124
Dysentery bacilli, Shiga, Flexner	+			+	122, 124, 125
Tubercle bacilli	+		+		123, 126 to 128
Diphtheria bacilli	+	Numerous (*a*)			129, 130
Meningococci	+	None			131
Pseudomonas aeruginosa	+			–	132
Streptococci	+	Group-specific			133
Streptothrix sp	+				129
Spirochetes	+		+	–	See text
Trypanosomes	+	(*b*)	+	–	51, 134, 135

(*a*) Streptococci, staphylococci, colon, tubercle and pseudodiphtheria bacilli, *Proteus* X_{19}, and *Bacillus subtilis*

(*b*) Negative for *Treponema pallidum*

evidently rich in the Wassermann-type antigen (134, 135), but their antisera do not react with spirochetal suspensions (51)

Alcoholic extracts of microörganisms differ, as a rule, in one essential point of behavior from alcoholic extracts of organs they are fully antigenic This means that they evoke antibody formation if introduced in the animal body alone, without the presence of a conveyor, and this is also the case with spirochetal extracts It is thus necessary to avoid bacterial contamination when such antigens are used for immunization

The role of bacteria and vaccine virus used recently in connection with the preparation of antisera to brain emulsion still remains to be determined (42, 58) [4]

Availability of antigens In the representative case of antibody formation against antigens of the Wassermann type, alcoholic extracts in combination with a conveyor are used The employment of suspensions or aqueous extracts of an organ will not cause antibody formation against the Wassermann-type antigen which is present in alcoholic extracts, but merely against species-specific (and often also organ-specific) substances, probably proteins The same is true of sediments obtained from saline tissue-extract by high-speed centrifugation (222) Furth and Kabat conclude that the Wassermann antigen is contained in a large complex molecule In any case, it appears that these peculiar antigens are usually not available in the cell in a form that is antigenically effective, as if such antigens were masked A quite instructive case of this type is the behavior of blood serum (137) Its introduction into a rabbit will not cause the formation of antibodies that react with alcoholic extracts However, an alcoholic extract of the serum injected together with the whole serum does evoke antibody formation of the Wassermann type (see 138) This observation may help to an understanding of why a substance in the body can be iso-antigenic It is also an important argument in explaining why substances in the diseased body (substances that are not antigenic under normal circumstances) may become antigenic In such a case, antigenicity might appear if in *infected* tissue a breakdown occurred that liberated the antigen

Not all substances contained in alcoholic extracts are as completely masked as in the example given above Antibodies against antigens extractable by alcohol are evoked by immunization with aqueous extracts from organs containing Forssman antigen, and also from such extracts containing the related blood-group A antigen The same is true for the antigen characteristic of malignant growth and for several bacterial antigens

[4] According to data published since this paper was finished, bacteria can be replaced by kaolin and other adsorbents Their role seems, therefore, to be merely a physical one (136)

The similar cases of syphilitic fetal liver and of Wassermann-type antigen in milk and yolk have already been mentioned

Competition of antigens The term "masked" antigen has been used, however, in another quite different sense, namely for the suppression of one antigen in a mixture of several present in an extract, suppression either of the antibody response, or of the antigen's *in vitro* reactivity, or of both Observations of this type have led to the hypothesis of the "competition of antigens" This notion is certainly incorrect as a generalization, but phenomena of this pattern are realities just the same (139 to 143) The basis of most of them is probably a difference in antigenicity or ease of combination In certain instances, chemical causes for this phenomenon have been suggested differences of solubility (144), formation of "micelles of antigens" (loose compound-formation) (145), and on the other hand, the breakdown of compounds (100)

Lack of protective properties Besides the antibody characteristic for the serum of man infected with syphilis, antibodies of this type seem to be evoked by tuberculous infection (146), and, less regularly, by malignant growths (see above) None of these antibodies seem to be associated with a protective mechanism This includes syphilis (147, 148) Contrary data lack confirmation, and recent evidence for some protective quality in syphilitic sera does not prove that the protection is due to the Wassermann antibody (29, 71)

The diseases just mentioned have in common the peculiarity that an effective resistance is not built up or is not of a predominantly humoral type The peculiar antigenic inertia of tissue spirochetes has been a matter of comment (149)

"Biological false reactions" and their theoretical significance The sero-diagnosis of syphilis has always involved precautions against the occurrence of non-specific positive reactions Technical progress has diminished this difficulty, but this problem needs to be discussed here because of its theoretical significance Non-specific reactions have been attributed in the past to what is called "increase of lability" in certain sera, but there has never been a satisfactory explanation of what is meant by the term

increase of the globulin content of such sera (frequently considered in the past to be the cause) has obviously nothing to do with the phenomenon, as there is indeed no correlation between the globulin content of the sera and their ability to react with alcoholic tissue-extracts (see for instance 150, 151)

More recently another explanation has found its expression in the term "biological false reactions" This expression implies that non-specific reactions might be caused by the presence in the serum of antibodies of related qualities which cause cross reactions because of similarities in their antigenic counterparts It would be unique indeed, if the production of antibodies of the type of the Wassermann antibody were entirely restricted to syphilitic infection As it is, it is peculiar enough that only in syphilis is this antibody response strongly and regularly manifested, at least in man It is, however, becoming increasingly clear that a fundamentally similar antibody formation can be engendered by various other disturbances of human physiological equilibrium, and it is necessary to take full cognizance of this situation in order to arrive at a deeper understanding of the problem at hand

Reports on "biological false reactions" have specified a number of infectious diseases in which such observations (152, 153) have been made malaria, leprosy, streptococcal endocarditis (154), measles (155), infectious mononucleosis (46), diphtheria (52), and even after smallpox vaccination (223) Yaws and pinta (or cavate) (219) are omitted from this enumeration because of their peculiar relation to syphilis, which makes it likely that immunological relations are very close (153) Recurrent fever, another human spirochetal disease, does not lead to a positive Wassermann reaction with any degree of regularity The occurrence of antibodies of similar type in tumoral diseases and their relation to embryonic growth have been discussed above, and it is only necessary to recall these facts in order to understand that "biological false reactions" in pregnancy and tumoral diseases belong in the same category

It may well be that one or another item of the incomplete list given above is included as a result of imperfect technique, just

as a much larger number has been eliminated for similar reasons in the past, but it has become increasingly certain that "biological false reactions" are not entirely to be eliminated by improvement of method Moreover, such false reactions cannot be eliminated by confirmation reactions based upon cleavage of the antibody-antigen complex (65, 66)

Fortunately these "biological false reactions" are of low titer as a rule Moreover, their interference with specific reactions in diagnostic tests for syphilis can be greatly diminished by modifying the technique in such a way that small amounts of antibody do not react with the antigen Unavoidably, this increase in specificity has to be paid for by elimination of low-titer syphilitic reactions The gain in safety thus achieved represents the most significant advance in the technique of the serological diagnosis of syphilis, as a highly competent observer has remarked (152)

"Normal" antibody of the Wassermann type In connection with the problem of false reactions, it might be mentioned that complement-fixing antibodies against cholesterol have been reported to occur in several human diseases (156) and that complement-fixation with lecithin has been seen frequently in monkey sera (157) One wonders whether the so-called non-specific reactions of rabbit sera (see above) may not some time find a similar explanation Positive Wassermann and flocculation reactions have been recently reported in a considerable percentage of sheep (158) and beef (224) sera

Role of tissue-specific antibodies in pathology The finding of organ-specific antibodies has aroused speculation as to their possible bearing in pathology The recent report (159) on kidney antibodies and their relation to nephritis is one example, another is the possible relation of syphilitic brain diseases to brain-specific antibodies (160) However there is no experimental evidence as to the latter In view of the pronounced organ-specificity of the Wassermann-type antigen from the brain, brain-specific antibodies have been expected to be found in the spinal fluid of patients with syphilis of the central nervous system However, reports of such findings could not be confirmed (46, 161, 162), and the reasons for this failure are unknown There is no suggestion of damage to the brain from the experimental introduction

of Wassermann-type, anti-brain antibody (46, 163) There is also no evidence as to the role played by tumor-specific antibodies (164) In this matter there might enter the old observation (165) that there are special mechanisms which tend to minimize the effect of complement upon the tissue (red blood cells) of the same species

THEORY OF SPIROCHETAL ORIGIN OF WASSERMANN ANTIGEN

Basic observations Wassermann's original conception that the antibody in syphilitic sera is a truly antispirochetal substance was abandoned by him after a bitter struggle It experienced, however, an unexpected resurrection when it was found that alcoholic spirochetal extracts are antigenic and are suitable antigens for practical diagnostic purposes It will be readily acknowledged that the idea possesses the advantage of simplicity, when presented in its modernized form which recognizes that the antibody is certainly not of the type commonly encountered in infectious diseases The experimental evidence for this hypothesis rests in the main on the observation of the formation of Wassermann-type antibody in rabbits and in man, after injection of spirochetal antigen (23, 46, 162, 166 to 168) However, this evidence is considerably less convincing than appears at first sight

Culture of spirochetes The most competent students of the biology of *Treponema pallidum* do not feel that cultivation of this microorganism in the test tube has ever been actually achieved (169, 170) The decision is a difficult one, because whatever it is that has been cultivated, infectivity for the rabbit and monkey is lost at once Spirochetes morphologically very similar to *Treponema pallidum* occur under conditions which make it appear quite possible that they may have been substituted during transfer from the syphilitic lesion Certainly, those spirochetes that are in use in Germany for the production of vaccines for experimental purposes and sold commercially as a diagnostic antigen are not likely to be *Treponema pallidum* (166, 169, 171) [5] This in itself may not be objectionable, because it is quite possible

[5] Culture spirochetes seem to be antigenically homogeneous (189) with the possible exception of one strain (190)

that the antibody formed in the case of immunization with spirochetal antigens is group-specific rather than specific for *Treponema pallidum* (168, 174)

Objections to the available evidence Whatever they may be, the spirochetes that are used as above have to be cultivated in media containing serum with or without tissue Suspensions made from such media may very well carry antigenic material derived from the medium, and not from the microorganisms (57, 172), in sufficient quantities for eliciting antibody formation Even washing the suspension, if done, does not preclude the retention of small amounts of antigenic matter from the medium

There were, of course, controls made with the medium alone (173) or media inoculated with bacteria (174), for instance colon bacilli, which did not lead to formation of the antibody in question But these controls cannot be relied on for two reasons (*a*) the uninoculated medium was not subjected to the metabolic action of the spirochetes, which may quite conceivably lead to the liberation of originally masked antigen (57), just as the alcoholic extraction does in the case of the serum referred to above (61) (*b*) Moreover, media inoculated with bacteria may not be subjected to the same kind of breakdown that is caused by the spirochetes (57) It cannot be overlooked in this connection that the conception of the Wassermann reaction as caused by an antigen derived from the body necessarily postulates an action of the spirochetes upon the cells of the body different from that caused by other pathogenic microörganisms This is implied in the very fact that the Wassermann reaction is characteristic for syphilis and no other disease (The admission of the possibility of Wassermann-type antibody formation by other causes would modify this statement only in the sense of a difference in degree and not in kind)

It must be pointed out that many of the spirochetal antigens used in experimental work contain phenol The importance of this compound for the practical value of the antigen was stressed by the originator of a widely used preparation (173, 175) It was mentioned before, that phenol acts not only as a fortifying agent (*Verstärker*), but it is supposed to set free the masked antigens

Thus, the value of all experiments using phenolized antigens for proving the spirochetal nature of the Wassermann reaction is doubtful

Differentiation of spirochetal and tissue antigens There is suggestive evidence that the Wassermann reaction and spirochetal complement-fixation occur independently (97, 176, 177) This is not necessarily proof of a complete difference in the antibodies, because similar observations can be made with any two Wassermann extracts Variations in the content of antigen and in its chemical properties could well account for differences Differentiation of the spirochetal and the Wassermann antibody by means of absorption has been repeatedly claimed (166), but others have not succeeded (168, 178)

There is a conflict of evidence on one important phase of this problem According to some observers, sera obtained by immunization with both spirochetal extracts and spirochetal vaccines can react to a similar degree with both alcoholic spirochetal extracts and alcoholic tissue-extracts (179, 180) Others, and they are in the majority, with the best controlled experiments, found spirochetal antisera to react solely or quite predominantly with spirochetal extracts (43, 46, 87, 100, 116, 168, 181, 182) This latter is the case especially in the reports concerning human beings treated with spirochetal vaccines for the double purpose of non-specific (fever) therapy and experimentation on antibody formation (167, 179, 183, 184) The same relation has been reported for skin reactions in human beings (183)

Cross reactions with brain-extracts have been described for such cases where immunization was effected by mixtures of spirochetal extracts and hog serum (100, 162) It has been suggested that this might have been due to the presence of cholesterol in both antigens Spirochetal extracts are markedly increased in their reactivity *in vitro* by cholesterol (100)

Trypanosomal Wassermann reactions Reactions clearly of the Wassermann type can be obtained from both animals and man immunized with trypanosomes (51, 135) This, however, remains an interesting parallelism with no conclusive value for the problem of the spirochetal origin of the Wassermann reaction Anti-

trypanosomal sera, as mentioned before, do not react with spirochetal antigens (51)

There is another fact that should be kept in mind, spirocheticidal effects, so readily obtained after experimental immunization, have not been observed in the case of actual infection either of man or of experimental animals (185)

The choice between the theory of tissue origin and that of spirochetal origin of the Wassermann antigen appears to depend on deduction rather than on direct evidence In view of the data presented above on the ubiquitous occurrence of the Wassermann antigen and its congeners, it would not be surprising to see the Wassermann antigen isolated both from tissue and from spirochetes In this case, a decision between the two competing theories could, of course, not be made with chemical methods, and it is difficult to see how it could be made by an immunological approach

It should be stressed that the uncertainty concerning the origin takes nothing away from the established facts on the immunological and chemical peculiarities of the Wassermann antigen

CHEMISTRY OF ANTIGENS OF THE WASSERMANN TYPE

Haptens of known composition Before entering the problem of the chemical nature of Wassermann-type antigens, brief mention should be made of the substances other than proteins that have been found to possess antigenicity The best known examples are the polysaccharidic substances isolated from bacteria or other cellular sources So far as these substances are composed solely of sugars and amino sugars, they possess no, or only incomplete, ability to evoke antibody formation, they are, however, reactive *in vitro* Full antigenicity is found, however, in those cases where polysaccharides occur as complexes with fatty acids, and phosphorus- and nitrogen-containing compounds as is the case in gram-negative bacteria of the coli-dysentery-salmonella group, (*antigène complet*) It is likely that the phosphatides isolated from tubercle bacilli are chemically constituted in a similar fashion They are also fully antigenic The component which determines the specificity is in either case the polysaccharide complex The "lipid" moiety is not antigenic

Relatively simply constructed substances have been found to be antigenic in the sense of being haptens which need a conveyor for eliciting antibody formation They are by this property linked to the group of the Wassermann-type antigens

The first reports on the hapten quality of such substances concerned lecithin and cholesterol (186) These experiments were, however, made on commercial preparations of low purity so that there remained doubt as to whether the antibodies observed were really against cholesterol or lecithin[6] Whereas the reports concerning the antigenicity of commercial products (187, 188, 192)[7] and similar preparations of cephalin and cerebrosides (188) have been confirmed, purified lecithins and cephalins from egg and brain have been found to be non-antigenic (187, 193 to 195), and lecithin from soya beans of doubtful antigenicity (195) On the other hand, antibody formation was obtained against a synthetic lecithin, the tristearyl-lecithin synthesized by Gruen (196, 197), and against recrystallized cholesterol and several congeners (di-hydrocholesterol, cholesterin oxyde, ergosterol (191, 196, 198)) Objections (199) to the validity of these observations from the point of view of the small amount of complement fixed are the less valid, because cholesterol and its derivatives do not only give complement-fixation of high specificity (196, 198), but also are flocculated specifically by their corresponding antisera (200) It has been found that *not* all cholesterol derivatives have hapten quality, (191, 201) oxycholesterol, cholesteryl bromide and several cholesterol esters seem to be devoid of this property Highly purified cerebron and lignoceryl-sphingosine are non-antigenic, whereas a polydiaminophosphatide from spleen, lung and liver has been shown to be a highly effective and specifically reacting hapten (202)

Quantitative considerations (69) show that the synthetic antigens are both less antigenic *in vivo* (as measured by the

[6] Low purity of testing material might explain in part the observation of odd cross reactions between lecithin and cholesterol and the respective antisera (186) However, similar cross reactions have been observed with lecithin free of cholesterol admixture, for which no explanation has yet been offered (191)

[7] Ovotellin, a full antigen, has been separated from crude egg lecithin (191) However, no effort has been made to determine the true relation of this antigen to lecithin antisera by the absorption test

amount of material and the duration of treatment necessary to evoke antibody) and *in vitro* (as measured by the minimal effective amount) than are natural antigens of the Wassermann type (see above)

These observations tend to show that substances of well-known constitution are able to act as antigens in a way similar to those observed in natural ones of the Wassermann type They increase the range of materials in which to look for substances of hapten nature They make us aware that substances of this type may be on the borderline between haptens and non-haptens, so that small chemical differences may decide whether they can produce specific antibodies or not In addition, they open an interesting field for the study of specificity and other problems of immunochemistry, until now little explored

Chemistry of tissue antigens There remains to be discussed what little is known about the chemistry of the antigens of the Wassermann type The active substance represents only a small fraction of the total extracted by alcohol The antigen is carried down from alcoholic extracts by cadmium chloride (203, 204) (Precipitates obtained (204) in this way are still used for diagnostic purposes) However, the conclusion that the Wassermann antigen is a lecithin, has not been borne out by further investigation (220) It was also realized early that the antigen is insoluble in acetone (204) It is a question whether the substance is really alcohol-soluble, because it has been demonstrated (205) that both the Wassermann and the Forssman antigens can be adsorbed from alcoholic solution by kaolin and other inorganic adsorbents and eluted into water or saline to give clear solutions (20, 206)[8] It may be that the alcohol-solubility at primary extraction is due to co-solubility caused by the presence of other substances, but this remains to be proved

The Wassermann antigen is recently reported (222) to be sedimented from saline tissue-extracts by high-speed centrifugation (27,000 rpm for one hour) together with organ-specific antigens, Forssman antigen, and several enzymes However, injection of

[8] Elution is also possible in organic solvents like benzene (209) Further data on adsorption and elution are given in references 206, 209 to 211

these sediments into rabbits does not cause formation of antibodies of the Wassermann type, whereas other antibodies, e g , that of the Forssman type, *are* evoked A logical interpretation of this observation would be that in these sediments (and presumably also in the intact tissue) the Wassermann antigen is hidden within large complexes and that for this reason its antigenic activity is not exerted (i e , masked, see page 305) Alcohol extraction would then be needed in order to liberate the Wassermann antigen from its connection with other material and make it effective as an antigen If such a conception is true, a similar effect as that of the alcohol could be surmised as becoming effective in diseased tissue Such a conception would be valid regardless of whether the complex from which the antigen is liberated is derived from the cells of the body, from spirochetes, or from both

A combination of the methods of acetone precipitation and $CdCl_2$ precipitation results in preparations that are effective in amounts of the order of one microgram (68, 207) Further elimination of ballast substances can be achieved by adsorption followed by elution into boiling methyl alcohol (208) Preparations obtained by the $CdCl_2$-acetone method have been found (68) to be non-reactive if tested immediately, but fully reactive after standing at room temperature for a few days[9] These purified antigens are not dialyzable (214), and hydrolysis destroys their activity (68, 215, 216) Analytical data on the purest preparations obtained up to now are given in table 2

All of these materials are not only reactive in complement-fixation and in flocculation with both rabbit and human (syphilitic) sera (68, 213), but they retain their full hapten quality of evoking antibody formation (213) They retain, so far as is known, their partial or total organ-specificity Work along similar lines has been done with alcoholic extracts from brain (214) and from tumor (216)

Another method (217) of purification takes advantage of the solubility of the antigen in acidified 80 per cent alcohol when shaken together with petroleum ether By a combination of this

[9] Is this an oxidative effect? There are other indications of the influence of oxygen on alcoholic tissue extracts (205, 212)

method with adsorption and elution, preparations have been reported with a purification factor of 100, but analytical data on these are not available as yet Further details on this phase of the subject cannot be given without a disproportionate amount of discussion beyond the limits set for this review

Recently, the non-protein part from floccules of syphilitic serum and cholesterinized tissue extract (Kahn) has been analyzed (221) The results, so far as they go, fit well with the data given above

A protagon from the brain has been isolated which contains 2 4 per cent N and 0 5 per cent P and which can function as a

TABLE 2

Analytical data on the purest preparations hitherto obtained

ANTIGEN	NITROGEN	PHOSPHORUS	SULFUR	FEHLING (CARBOHYDRATE)	FATTY ACIDS	REFERENCE
Heart	Little	+	0	0[a]		(68)
Brain	1–2%	0		2%[b]	+	(214)
Tumor	0	0		Very little	+[c]	(216)
Forssman	2–2 3%[d]	0 1%[e]	0 17%[e]	30%[f]	Probably +	(67)

[a] 7% by reduction of potassium ferricyanide (214)
[b] Probably 50% as creatinine
[c] Cholesterol ester +
[d] ½ or more as hexosamine
[e] From contaminants ?
[f] Aldohexose Total C = 60 to 70%

hapten (218) Its antiserum has been found to give cross reactions with aqueous but not with alcoholic extracts from brain, and with lecithin prepared from brain This serum did not react with alcoholic extracts from other tissues, nor with lecithin prepared from egg, nor with cerebron or sphingomyelin

The method of tryptic digestion and alcohol fractionation and the trichloracetic acid method, both successfully employed for the isolation of the "antigène glycido-lipidique" of gram-negative bacteria, have not proved effective with spirochetes (168)

It is difficult to draw any conclusions as to the nature of the effective material from the data available at the present time

Nor do they give any additional indication as to whether the antigen which causes the formation of the Wassermann antibody in syphilitic man is furnished by the spirochete or by the host

CONCLUSION

Owing to the scantiness of biological and especially of chemical data the immunological aspect necessarily is predominant in this review

Syphilitic infection of man and animals causes a production of antibodies directed against one or more closely related substances of a peculiar kind Immunological experimentation has further substantiated the antigenic qualities of these substances The mere fact of their rather ubiquitous distribution should arouse general biological interest which has so far taken cognizance of the Wassermann antigen only in a perfunctory way

The observations derived from and concomitant to experimentation concerning the Wassermann reaction have led to the detection of substances of similar immunological behavior but differing from the Wassermann antigen in more pronounced specificities and in being more limited in distribution The existence of chemo-specific and organ-specific antigens has been established The investigation of the significance of these antigens (and their corresponding antibodies) is only in its beginning

We are faced with a maze of interrelated substances which are carriers of complicated and subtle specificities This explains why their serological differentiation is often beset with difficulties The simultaneous occurrence of multiple specificities within one tissue or one kind of cell and the corresponding formation of multiple antibodies upon immunization have been found to be frequent The degree of antigenic predominance probably depends not only on quantitative relations, but also on factors such as solubility, adsorption, and complex formation which are all hidden behind the term availability The determination of all of these factors will be a necessary part of future work in this field

Similarities in immunological behavior form the common link of this group of substances They are all extractable by organic

solvents from tissue and other organized matter[10] They have been designated in this review as Wassermann-type antigens Nobody is more aware than the author of the preliminary and provisional nature of this classification and its need for verification and rectification by chemical methods In taking stock of the present state of our knowledge in this matter, it is hoped that attention will be drawn to a field invitingly open for further investigation

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Note—This review covers publications available up to September 25, 1941 Citation of the great number of communications on the many experimental details is not permissible owing to limitations of space In many cases, the selection of references is made not so much from the standpoint of priority of publication as for giving access to sources of details Where the work of one laboratory is reported in a series of communications, the most recent publication containing references to prior work is given preference for quotation

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THE FILTRABLE MICROÖRGANISMS OF THE PLEUROPNEUMONIA GROUP

(APPENDIX TO SECTION ON CLASSIFICATION AND NOMENCLATURE)

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The publication (1941) of my suggestions for the classification and nomenclature of the filtrable microorganisms of the pleuropneumonia group has brought forth a number of important comments It may be desirable, therefore, to extend the original discussion because many points have been raised which may ultimately have to be considered by the International Committee on Bacteriological Nomenclatures The various comments, in general, fall into the following categories

1) "Any attempt to classify taxonomically these pleuropneumonia-like organisms is sure to be premature as we do not yet fully understand the cycle of development of any of them in artificial culture" (R St John-Brooks, J C G Ledingham, and E Klieneberger)
2) Taxonomic classification is advisable and needed but that the one suggested is contrary to the rules of priority in nomenclature and to the principle of naming the family from the type genus, and the order from the type family (R E Buchanan)
3) That the properties and characteristics of the class *Schizomycetes* need not necessarily exclude the microörganisms of the pleuropneumonia group (R E Buchanan)
4) That the attempt to classify microörganisms into genera according to the host of origin has not found favor in the past (R S Breed and R St John-Brooks)
5) That *nomina hybrida* have been used

Since the comments about the laws of priority and principles of nomenclature are the only ones in which it is possible to appeal

to rules, it may be advisable to review the various attempts at nomenclature of the pleuropneumonia group During the years that the microörganism of bovine pleuropneumonia remained a unique individual in the microbial world it received many names which varied with the author's concepts of its nature Thus, while Bordet (1910) was the first to recognize its morphological complexity and considered its relationship to vibrios and spirochetes, Borrel *et al* (1910), believing it to be essentially of coccal nature, devised the binomially correct *Asterococcus mycoides* As other investigators differed with the morphological concept of Borrel *et al*, new names were proposed as follows

Coccobacillus mycoides peripneumoniae (Martzinovski, 1911)
Micromyces peripneumoniae bovis contagiosae (Frosch, 1923)
Mycoplasma peripneumoniae (Nowak, 1929)
Asteromyces peripneumoniae bovis (Wroblewski, 1931)

It may be pointed out, however, that, quite aside from the question of priority, three of these names are invalid because they were proposed as polynomials, and only *Mycoplasma peripneumoniae* (Nowak) would require consideration in the event that *Asterococcus mycoides* proved to be invalid In 1931, Wroblewski named the new member of the group, the infectious agent of contagious agalactia of sheep and goats, *Anulomyces agalaxiae* In 1933, Ledingham referred to the confusion of names and suggested that the two microörganisms known at that time be placed in the order *Actinomycetales* and in the family of *Actinomycetaceae* While he suggested that a new genus be set up to include the microörganisms of bovine pleuropneumonia and of agalactia of sheep and goats as species, he thought that the selection of an appropriate name should await further consideration and research Turner (1935) disagreed with Ledingham and proposed the new order *Borrelomycetales*, family *Borrelomycetaceae*, genus *Borrelomyces* In 1936, Shoetensack named the new microörganisms from dogs, *Asterococcus canis* Since then many new related microörganisms have been isolated and the existence of a distinct microbial group became apparent In 1939, however, the editors of the 5th edition of Bergey's Manual of Determinative

Bacteriology decided to leave out *Asterococcus*, among certain other previously recognized genera, because "the organisms included in these genera are little known and they have not been generally recognized in later classifications"

The classification and nomenclature of the pleuropneumonia group which I suggested is the first one based on an analysis of the numerous species which have come to light in recent years, and I should deem it unfortunate indeed, if the laws of priority would "freeze" the many new names that I have suggested despite the fact that they may be out of place in a more reasonable classification that may be devised later on The following considerations formed the basis of the suggested classification and nomenclature

1) The microörganisms of the pleuropneumonia group can be regarded as sufficiently distinct from the bacteria to be dealt with in a separate class
2) They are readily distinguished into parasitic and saprophytic groups
3) The parasitic group can further be differentiated into genera which remarkably enough vary with the host that is parasitized
4) A number of distinct species may exist in a single host, thus providing a varying number of species for each genus

The divisions were made and the names selected with these considerations in mind, because they seemed to supply a more easily remembered and logical scheme than that in which the names of the family and order would have been derived from the name of the type genus (The order *Eubacteriales* appears to have been established without derivation from below, i e , from a type genus and family) Similarly because the Latin roots of dog, rat, mouse, etc are more familiar than the Greek, it seemed to me to be one instance where hybridization might be helpful and perhaps even desirable

While I consciously deviated from custom in suggesting this scheme, I must confess that I failed to realize the real legal claim of *Asterococcus mycoides* and the influence which that would have on any classification of the "pleuropneumonia" group The main objection that I have to the name *Asterococcus mycoides* and to

deriving the names of the family and order from it, is that in intimating a coccal nature for the group it not only gives a misleading concept of the essential characteristics of these microbes, but also tends to create confusion by suggesting a morphological relationship to other cocci which does not exist However, according to Art 15 of the *Rules of Nomenclature*, "the purpose of giving a name to a taxonomic group is not to indicate the characters or the history of the group, but to supply a means of referring to it," and according to Art 59, "A name or epithet must not be rejected, changed, or modified merely because it is badly chosen, or disagreeable or because another is preferable or better known" Furthermore, it is doubtful whether even a broad interpretation of Art 63 which says "A name of a taxonomic group must be rejected when its application is uncertain" could be invoked in this case

Should it finally be decided that the name *Asterococcus mycoides* cannot be invalidated, my inclination would be to select *Anulomyces* (from *Anulomyces agalaxiae*, Wroblewski, 1931) as the type genus and to reconstruct the original classification in accord with the rules of priority and accepted principles of nomenclature as follows

Class *Paramycetes*
  Order I *Anulomycetales*
    Family I *Anulomycetaceae*
      Genus 1 *Anulomyces*
        Type Sp *A agalaxiae*
      Genus 2 *Asterococcus*
        Type Sp *A mycoides*
      Genus 3 *Canomyces*
        Type Sp *C canis*
      Genus 4 *Murimyces*
        Type Sp *M pulmonis*
      Genus 5 *Musculomyces*
        Type Sp *M neurolyticus*
    Family II *Sapromycetaceae*
      Genus 1 *Sapromyces*
        Type Sp *S laidlawi*

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FACTORS INFLUENCING BACTERIAL GROWTH IN BUTTER[1]

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From the standpoint of suitability for bacterial growth, butter differs from other dairy products In butter the water is not the continuous phase, as it is in milk and cream, but is dispersed throughout the fat as small droplets The fat, which makes up approximately 80 per cent of butter, is relatively resistant to bacterial action, although certain species may attack it With the water droplets walled off by fat, bacteria cannot migrate from one droplet to another, and for the most part growth is restricted to the droplets originally infected The water in butter contains different food materials, including proteins, lactose and salts, that are satisfactory for growth of various bacteria However, in the case of salted butter the water also contains added sodium chloride which delays or prevents growth of many organisms With salted butter the salt content varies widely, and actually there is less difference in salt content between unsalted and lightly salted butter than between lightly and heavily salted butter

The presence of water and materials in solution or suspension in it makes butter much more susceptible to bacterial action than are various edible fats and oils that contain very little water and practically no food elements other than fat Even with its susceptibility to bacterial action, butter commonly is expected to keep well, although at times it is subjected to comparatively high temperatures for rather extended periods

Butter regularly contains bacteria arising from various sources, such as the cream, equipment, water used to wash the butter, air, employees, cultures added to give the butter a desirable flavor

[1] Journal Paper No J-263 of the Iowa Agricultural Experiment Station, Ames, Iowa Project No 119

and such miscellaneous sources as salt, packaging materials, etc , with rare exceptions the cream is pasteurized, but some survival of bacteria is to be expected with the usual pasteurization procedures Many of the bacteria from these various sources grow in butter unless prevented by the holding temperature, salt concentration or some comparable factor Growth of certain of the organisms results in flavor defects in the butter For the most part the organisms responsible for these defects are easily destroyed by heat, and they come commonly from contaminated equipment, with which the cream or butter comes in contact following the pasteurization, or from defective water supplies

PHYSICAL CONDITION

The water in butter is in the form of small droplets and it has been recognized that this is a significant factor in bacterial growth in butter In all probability the situation is different with bacteria than with yeasts and molds

In an extensive study of mottled butter, Storch (104) found that the water therein consists of a very large number of finely divided droplets Hunziker and Hosman (42) confirmed this report and noted that addition of salt causes a reduction in number and an increase in size of the water droplets, thus giving the butter a deeper yellow color than it had before salting Boysen (4) reported that the numbers of water droplets in butter usually range from about 11 to 18 billion per ml , working the butter increases the number of small droplets and decreases their tendency to aggregate, while salting decreases the number of small droplets and increases their tendency to aggregate

The effect of the distribution of water in butter on growth of bacteria was considered by Rahn and Boysen (82) Since the numbers of water droplets in butter approximate 10 to 18 billion per g , while the numbers of bacteria are always much less than these values, many of the droplets must be sterile The volume of water that is free of bacteria depends on two factors, the number of bacteria in the cream at the time of churning and the degree of water dispersion in the butter As evidence of lessened bacterial activity with increased working, it was found that acid

production in butter serum was less with over-worked than with normally worked butter It was suggested that the thorough working of butter improves the keeping quality

Hammer and Hussong (32) noted that all the nutrients in butter are not available for bacterial growth When the serum of unsalted butter was separated from the fat, development of bacteria was more rapid at either 7 or 21 C than when the product was in the normal physical condition Collins and Hammer (8) reported that migration of bacteria through butter is not common, and when it does occur poorly worked butter is usually involved

Using an adaptation of Burri's smear-culture technic, Long and Hammer (54) examined very small portions of butter for their bacterial contents An irregular distribution of the bacteria was noted, and in many instances the variations were extreme This was considered additional evidence that bacterial development in butter is largely limited to certain focal points, probably infected water droplets

Knudsen and Jensen (48) demonstrated that bacterial activity in butter decreases as the working increases, they believed that working tends to decrease the size of the water droplets, thus reducing the nutrients available for growth Later, they (49) noted that after storage butter with many small water droplets was more satisfactory than butter with large droplets Conditions which tended to give large droplets, such as addition of salt and churning of high-acid cream, resulted in butter of poor keeping quality For estimating the number and size of free water droplets in butter, a rapid method employing indicator paper has been proposed by Knudsen and Sörensen (50)

Scharp (94) concluded that, as the working of commercial unsalted butter is increased, the number of organisms after any holding period decreases, this was attributed to finer dispersion of the water As judged by rate of growth, production of defects, production of acid by butter culture organisms and production of fatty acids by lipolytic organisms, Long and Hammer (55) found that bacteria were more active in under-worked than in thoroughly worked butter Pont (74) also emphasized that the

physical structure exerts considerable influence on bacterial activity in butter

The effect of the physical condition of butter on development of the putrid defect has been investigated in some detail Cullity and Griffin (9) and also Pont (75) noted that thorough working of butter retarded the appearance of the defect Itzerott (43) observed that the rabbito defect occurred most frequently and developed most rapidly in butter showing an open texture and free moisture Conversely, Totman, *et al* (105) found no convincing evidence that the amount of working which butter receives has an appreciable effect on the keeping quality, they stated that very seldom is butter worked so little that the water and salt are not sufficiently distributed to provide the benefit of the antiseptic action of salt

Since the amount of working that butter receives at the time of manufacture has an effect on growth of bacteria in it, working subsequent to manufacture also would be expected to affect the growth Prucha and Brannon (78) found that butter which had gone through a printing machine having a crushing action did not keep as well as butter not so treated, in one case a decided off flavor quickly developed in the printed butter, while the unprinted butter did not develop the defect Gratz (24) observed that bacterial growth was greater in the outer layers of butter than in the interior and emphasized the importance of this in the lipolysis of reworked butter In several comparisons involving butter reworked with and without the outer layer removed, it was found that fat hydrolysis was more pronounced without the removal Probably redistribution of organisms through the butter by the reworking was a factor of significance Cullity and Griffin (9) reported cases in which the reworking of apparently sound butter rapidly resulted in the putrid defect Turgasen (106) cited instances in which butter printed in a machine having a reworking effect became cheesy, while unprinted butter from the same churning kept satisfactorily

Long and Hammer (56) concluded that the reworking of unsalted butter, made from pasteurized cream inoculated with various organisms, frequently increased the activity of the organisms at 10 C , as evidenced by growth rates, production of defects,

decreases in pH values of the butter serum when butter cultures were added to the cream and increases in acidities of the fat when lipolytic organisms were used Apparently redistribution of organisms and aggregation of water and the nutrients contained in it were primarily responsible for the increased activity In some instances the reworking of salted butter containing organisms capable of producing defects has tended to favor development of the defects, while in other instances reworking has had no effect on deterioration or even tended to inhibit bacterial action (36)

Results of various investigations indicate that the physical condition of butter has a marked influence on growth of bacteria in it *With unsalted butter there appears to be a definite relationship* between the amount of working that the butter receives and bacterial growth, with salted butter the same general relationship probably obtains, although the dispersion of the water may be affected somewhat by the presence of salt The reworking of butter under certain conditions tends to accelerate bacterial growth at favorable temperatures

TEMPERATURE

Just as it influences the action of bacteria in various food products, the temperature at which butter is held would be expected to influence the activity of bacteria therein Low temperature constitutes one of the important factors in the control of both bacterial and chemical deterioration of butter

Bacterial changes in butter held below 0 C , where little change would be expected, have been repeatedly investigated Sayer, *et al* (93) found that low temperatures entirely checked the growth of many species, while others grew only slowly It was noted that salt lowers the freezing point considerably and thus may interfere somewhat with the effect of low temperatures In certain samples the freezing point of the brine was -22 C Later Rahn, *et al* (83) reported that some microörganisms multiplied in salted butter at -6 C , moreover, salted butter kept better than unsalted above the freezing point as well as below

In salted butter made from raw and pasteurized cream plus

butter culture, Rogers and Gray (89) found that the numbers of bacteria gradually decreased at both −23 3 and −12 2 C, the decrease being slightly more rapid at the higher temperature Deterioration of high-acid butter at low temperatures was not attributed to microbiological action

Brown, *et al* (6) investigated changes in bacterial numbers in salted butter held at −17 8 C for 428 days and found that relatively high bacterial counts persisted in butter over 1 year old However, during the first 9 days of storage there was a rapid decrease in bacteria after which the decrease was more gradual Lactic acid organisms persisted as long as 275 days and in one case for 426 days It was suggested that low temperatures and salt decreased the rate of metabolism

Grimes (25) stored salted butter, made from pasteurized sweet cream plus 10 per cent butter culture with and without ripening, at −21 1 C for 6 to 7 months In the butter made from ripened cream, 95 to 99 per cent of the bacteria died during the holding In the sweet-cream butter, the average decrease in numbers of bacteria was 20 per cent, the maximum being 65 per cent, some lots showed no decrease When proteolytic bacteria were added to either type of cream before churning, they were not a significant factor in the deterioration of the butter

Arup and Gilmour (2) held salted butter made from unripened pasteurized cream at −7 C for 6 months Bacterial counts every 4 weeks indicated that little or no bacterial growth took place, nor was there any considerable reduction in counts The temperature of storage was sufficient to stop bacterial action regardless of the extent of infection or of the moisture, acid, curd, iron or copper contents Later, Arup and Gilmour (3) found that bacterial counts decreased in each of three lots of salted butter held at −12, −6, and −2 C

Nelson and Hammer (67) stored butter at −20 C following 7 days at 21 C There were decreases in bacterial counts whether the butter was salted or unsalted, the decreases averaging 64 per cent with salt and 74 per cent without salt In both types of butter the largest decreases in counts occurred with the samples having the highest counts on entering storage

Changes in the flora of salted sweet-cream butter held at −9 4 C for 2 to 8 months were investigated by Grimes and Hennerty (26) In general, increases in total counts (bacteria and yeasts) occurred The increases were attributed to the growth of yeasts rather than of bacteria There was neither a definite increase nor decrease in numbers of gelatin-liquefying bacteria during the storage

Loftus-Hills, *et al* (53) found only slight changes in bacterial numbers during storage of salted butter at −11 1 C for 3 months In general, lactose-agar plate-counts decreased, while gelatin plate-counts increased During the storage *Escherichia-Aerobacter* organisms, acid-producers and gas-formers disappeared in some samples but appeared in others

Changes in bacterial numbers in salted and unsalted butter held at −25 C were compared by Jacobsen (45) who reported marked reduction in numbers with both types of butter Apparently freezing destroyed the small numbers of proteolytic and lipolytic bacteria originally present Turgasen (106) investigated the effect of extended holding of butter at −28 9 C on the activity of organisms causing cheesiness, samples of infected print butter held for 6 months developed the defect when removed from storage and placed at 21 1 C

At 0 C or above, changes in bacterial numbers depend considerably upon factors other than temperature Some investigators have reported decreases in this temperature range, while others have reported increases In salted butter made from raw and pasteurized cream plus butter culture and held at 0 C , Rogers and Gray (89) noted decreases in bacteria In salted butter held at 0 to 10 C for 37 days, or at 35 C for about 1 month Lafar (52) also obtained decreases, at room temperature the bacteria increased rapidly at first and then began to die, leaving the butter rancid and tallowy Wilson and Prucha (113) found that 3 weeks at 18 3 C almost completely eliminated acid-forming organisms from salted butter, leaving alkali-formers and an inert group, the flora of butter stored at 4 4 C tended to remain the same as that of the fresh butter

Except for two organisms, Fennema (16) found that a tempera-

ture of 0 to 10 C largely prevented growth of the cultures employed in salted and unsalted butter made from sterilized cream, *Serratia marcescens* and an organism producing fishiness showed some slight growth in salted butter during the first few days of holding Guthrie, *et al* (29) investigated the changes in salted and unsalted butter held at 5, 10 and 24 C and found that all samples deteriorated more rapidly at the higher holding temperatures

In unsalted butter held at 21 C, Hammer and Hussong (32) noted rapid increases in numbers of bacteria, while at 7 C there also were increases but they were much less rapid than at 21 C In unsalted butter stored at 0 C, Macy, *et al* (61) found that bacterial counts usually were higher after 1 or 9 months than originally The increases were more frequent and marked in the butter held 1 month, usually the maximum count was reached in about 10 days, after which there was a gradual decrease In salted butter a large percentage of the samples had decreased in bacterial count after 1 or 9 months The unsalted butter frequently developed defects, while the quality of the salted butter remained reasonably satisfactory Shepard (96) reported that bacterial development in unsalted butter held 14 days was more rapid and extensive at 21 C than at 0 C

According to Garrison (21), fluorescent bacteria in unsalted butter survived 6 months at 1 to 3 C Slightly less than half the cultures studied produced flavor defects at these temperatures

Itzerott (43) suggested that temperature is the greatest factor influencing development of the rabbito defect At temperatures between 15 6 and 32 2 C the defect developed in 2 to 4 days, while at temperatures below 12 8 C development of the defect was retarded

Changes in flavor of butter under commercial cold-storage conditions (−10 C or lower) appear entirely uncorrelated with microbiological activity in all types of butter, according to Pont (74) In general, no convincing evidence of biological activity in butter under these conditions has been produced and, from theoretical considerations alone, none would be expected At temperatures above 0 C marked degradation of quality may be

brought about by bacterial development In this respect, particular organisms or groups of organisms appear of more significance than total bacterial populations Pont emphasized that under commercial conditions, butter commonly is subjected to temperatures which permit bacterial development both before and after periods of cold storage Normally, after manufacture at least 3 or 4 days elapse before the temperature of the butter falls below 5 C, often it may be above 0 C for 10 days or more before the butter is consigned to storage After storage, temperatures above 0 C again are used to permit grading, printing and disposal on the retail market

The data indicate that growth of bacteria in butter is largely prevented by temperatures of 0 C or below and that some destruction of bacteria may result However, it is possible that in salted butter growth of certain bacteria may occur at temperatures slightly below 0 C due to depression of the freezing point by the salt, such growth is limited both by the temperature and the salt At temperatures above 0 C bacterial development may be rapid, especially in unsalted butter

SALT

The retarding effect of salt on the activity of bacteria in butter is generally recognized and extensively used in butter manufacture, although some butter is made without salt Amounts of salt used in butter vary widely (from a fraction of 1 per cent to about 4 per cent) depending on the market for which the butter is intended Unsalted butter is of two principal types, that made with low churning acidities for use in reconstituting cream, etc and that made with high churning acidities so that it will have considerable flavor

General observations Lafar (52) reported that salt had a definite bactericidal action in butter held at 0 C for about 30 days, light salting (0 5 to 1 0 per cent) seemed as effective as heavier salting at both 0 and 35 C

In two lots of butter, one salted and one unsalted, held at 0 to 3 3 C, Loveland and Watson (58) found that the numbers of

bacteria gradually diminished as the butter aged, the decrease being much greater in the salted butter than in the unsalted There was a more rapid decrease during the first few hours of holding than later Bacterial counts on the fresh butter were extremely high, being 115,302,000 per g for the unsalted butter and 54,170,000 per g for the salted butter

Fettick (17) stored salted and unsalted butter in a refrigerator, which varied in temperature from 0 to 18 C , for 5 months During this period the numbers of bacteria in the unsalted butter were higher than in the salted at each examination In the unsalted butter the numbers were still increasing at the end of the holding, while in the salted butter the numbers increased for about 1 month and then did not change materially although some fluctuation was noted Fettick believed that butter with 2 or 3 per cent salt keeps better than that with higher percentages, the reason being that the lactic acid bacteria can tolerate the lower amounts but not the higher, if the lactic acid bacteria are killed by high salt content, other types which are not salt-resistant may grow and produce undesirable changes

Rahn, *et al* (83) found that salted butter kept better than unsalted, both above and below 0 C They also noted that there are microorganisms in butter which can multiply in salted butter at −6 C , whether these organisms could cause deterioration was not determined

McKay and Larsen (63) concluded that salt improves the keeping quality of butter Later, they (65) stated that, so far as the keeping quality is concerned, it would be advisable to salt butter as heavily as 6 per cent Such salting would tend to check deterioration due to bacteria Fennema (16) concluded that salt is a very important factor in butter preservation, he found that salt not only checks bacterial growth but quickly decreases the numbers of organisms in the butter

The Minnesota Agricultural Experiment Station (66) reported that salt affects butter by both the direct chemical and the indirect biological routes At temperatures limiting bacterial growth, the effect of salt is to deteriorate butter, under conditions favoring bacterial growth, salt tends to inhibit such growth and

preserve butter Unsalted butter kept better than salted at −12 2 C

Bacteriological changes in salted and in unsalted butter held at −26 C were compared by Washburn and Dahlberg (108) After 113 days every sample of unsalted butter contained more bacteria than its salted duplicate, and similar, though less uniform results, were obtained after 284 days Low temperature appeared to be so important in preventing bacterial development that the antiseptic property of the salt played a minor role *Streptococcus lactis* withstood the adverse conditions better than other organisms

Weigmann (109) recognized that salt is a common, but by no means powerful, preservative for butter and butter-like substances He observed that unsalted butter, when not made with special precautions, contains more microörganisms than salted butter and that the latter possesses the better keeping quality Spitzer, *et al* (103) studied changes in the flora of stored butter, the number of butter samples showing bacterial increases during storage decreased as the salt concentration in the brine increased Later, Spitzer and Parfitt (102) reported the same results and also that proteolytic bacteria were inhibited least by the salt in butter

Macy (60) noted a general tendency for salted butter to decrease in bacterial count during storage at 0 to 1 7 C for 1 to 9 months, while unsalted butter showed an increase There was no greater tendency for the more highly salted samples to decrease in count than for those of lower salt content, this was explained by the fact that the majority of organisms inhibited by salt are inhibited by relatively small amounts When the data were considered from the standpoint of concentration of salt in the brine, instead of in the butter, essentially identical results were obtained, although there was a slight indication that more concentrated brines were somewhat more inhibitory

Hammer and Hussong (32) reported that salted butter held at either 7 or 21 C tended to decrease in bacterial content Horovitz-Vlasova, *et al* (39) concluded that salting efficiently prevents bacterial growth in butter if the cream is salted before churning, but its power is limited by the low solubility of salt in butterfat

Loftus-Hills, *et al* (53) stored butter at −11 1 C for 3 months and found no relationship between salt content or brine concentration and keeping quality of butter, or between brine concentration and bacterial increase or decrease

Changes in the flora of salted and unsalted butters made from sweet cream plus 8 per cent butter culture and held at 0 C for 5 to 19 weeks were studied by Shepard (96) In the salted butter a gradual decrease in bacteria occurred, while in the unsalted there was an extensive increase

Effect of salt on development of defects and on growth of organisms The ability of salt to retard appearance of butter defects caused by microörganisms has been studied by various investigators The effect of salt on organisms encountered in butter, but not normally capable of producing defects, also has been considered

Schmidt (95) found that salt retarded the development of rancidity in butter but was less effective than pasteurization of the cream used for the butter The best keeping quality was obtained through a combination of salting and pasteurization Orla-Jensen (68) washed butter containing *Pseudomonas fluorescens*[2] with 25 per cent aqueous salt solution, so that the salt content of the butter was 2 9 per cent, and found the organism almost completely inhibited He noted that the butter contained 13 4 per cent water, which resulted in a 21 6 per cent brine, a concentration sufficient to prevent growth of almost all bacteria

According to Giltner and Baker (23), microorganisms which liquefy casein and gelatin are more easily affected by salt than some non-liquefiers Virtanen (107) reported that fermented, cheesy, putrid and rank flavors in butter generally are caused by organisms that are often of the water type and generally inhibited by salt

The percentages of salt in surface taint butter agree very closely with the percentages in normal butter, according to Hood and White (38) Surface taint was noted in butter with as high

[2] The name "*Bacterium fluorescens liquefaciens*" has been used by different investigators It is assumed to refer to the organism now commonly designated *Pseudomonas fluorescens* although additional classification studies on the fluorescent *Pseudomonas* organisms may change this idea

as 2 67 per cent salt, equal to a brine concentration of 15 37 per cent They questioned the control of the responsible organisms by high salting since many markets demand butter with less than 2 67 per cent salt

The effect of salt on development of surface taint at 15 6 C was investigated by Derby and Hammer (10) who found that unsalted and low-salted butter made from cream inoculated with defective butter developed surface taint in a short time, while medium-salted butter remained normal during the 7-day holding period Plate and direct counts after 2 and 7 days were higher on the defective butter than on the normal butter When *Pseudomonas putrefaciens* was used for inoculation, unsalted butter and low-salted butter were putrid in 4 days, while medium-salted butter was still normal after 20 days Claydon and Hammer (7) indicated that salt tends to prevent the putrid defect in butter but noted that many lots of commercial salted butter become putrid In studies on cheesy butter, Turgasen (106) found that as much as 4 per cent salt sometimes did not prevent the condition Pont (75) emphasized the value of heavy salting in controlling the putrid defect

Itzerott (43) reported that salt had a greater effect than acidity in checking growth of organisms causing the putrid condition in butter, a salt content of 1 7 per cent gave some protection Long and Hammer (57) noted that in experimental butter containing 2 5 per cent salt and held at 3 and 21 C growth of *P putrefaciens* was inhibited, after a slight increase in numbers early in the incubation period a decrease occurred Salting and working prevented appearance of a definite defect In unsalted butter growth was rapid at 3 and 21 C , and the putrid defect developed rapidly

In butter containing 2 per cent salt and held at 21 C , Nelson and Hammer (67) found that butter-culture streptococci developed little or not at all, while in unsalted butter the organisms developed extensively Jacobsen (44) reported that 2 5 per cent salt in butter prevented growth of lipolytic and proteolytic bacteria at −25, 21 C and temperatures between

Rice (85) noted that in salted butter at −10 C *Escherichia-*

Aerobacter organisms persisted for 8 weeks At 15 6 C the organisms lived for considerable periods, but their reproduction was almost suspended, after 21 days there was a slight increase in some samples and a slight decrease in others However, other microörganisms, especially chromogenic micrococci, increased in numbers In unsalted butter at 15 6 C , *Escherichia-Aerobacter* organisms increased very rapidly

White (111) investigated a black discoloration of salted butter and isolated the causative organism which he named *Pseudomonas nigrifaciens*, it is probably identical with an organism described by Hiscox (35) The salting of butter with at least 1 25 per cent salt, to give approximately 7 per cent salt in the serum, was suggested as a control measure

Hammer and Olson (33) reported that organisms which actively produced phosphatase in milk also rapidly produced it in unsalted butter, these included *P putrefaciens*, *P nigrifaciens*, *Pseudomonas mephitica* and *Flavobacterium fecale* In general, when the butter was salted, production of phosphatase was less rapid and less extensive than in the corresponding unsalted butter but was still definite with various organisms

Effect of salt on activity of organisms in bacteriological media Investigators have determined the salt tolerance of different organisms by growing them in liquid media or on solid media containing known salt percentages While many of the organisms studied are not of importance in butter, a rather exact knowledge of the salt tolerance of various organisms or groups of organisms is useful in considering the inhibitory effect of different brine concentrations in butter

McKay and Larsen (64) studied the effect of salt on a spore-forming organism and a gas-forming organism isolated from butter In a medium containing 4 per cent salt both grew, while in a medium containing 6 per cent salt neither grew

Pettersson (71) reported that the rod-shaped bacteria investigated did not grow in bouillon containing more than 10 to 12 per cent salt, while most cocci grew very well with 15 per cent salt present Putrefying bacteria were more sensitive to salt than other types The inhibitory action of salt was especially pronounced at concentrations of 20 to 25 per cent

The effects of salt on various organisms often found in butter were studied by Fettick (17) who indicated that the following types were quite sensitive to salt Lactic acid bacteria, *P fluorescens*, *Bacterium fragariae* (probably *Pseudomonas fragi*), *Alcaligenes viscosus* and *S marcescens* Organisms that were quite resistant to salt included *Escherichia coli*, *Aerobacter aerogenes* and certain spore-formers

Brown (5) studied the salt tolerances of organisms isolated from butter held in storage at −19 4 to −16 1 C Twenty-four of 57 cultures of bacteria grew on a 12 per cent salt medium at 20 C , and four of these grew well on the medium at 6 C The ratio of liquefying to non-liquefying cultures was much the same whether the bacteria were isolated on ordinary agar or on agar containing 12 per cent salt

Giltner and Baker (23) investigated the effect of salt on the flora of butter and found that 8 per cent retarded the physiological processes of most organisms, although concentrations of 12 to 20 per cent did not inhibit all growth, streptococci were sensitive to salt, while micrococci and staphylococci tolerated high percentages

Gubitz (27) noted that *P fluorescens* grew poorly in media containing 5 per cent salt Of two gram negative, fluorescent, gelatin-liquefying bacteria studied by Henneberg (34), one tolerated 7 5 per cent salt, while the other tolerated only 5 per cent Henneberg indicated that salt is one of the factors tending to protect the fat and protein of butter from the action of *P fluorescens* Of twelve species of the *Alcaligenes* group, one tolerated 15 per cent salt, four 10 per cent, five 5 per cent and two 2 5 per cent, of five species of streptococci, three tolerated 5 per cent salt and two 2 5 per cent, of eight species of micrococci, seven tolerated 15 per cent salt and one 2 5 per cent, and of three species of the *Proteus* group, one tolerated 15 per cent salt and two 10 per cent

The salt tolerances of some lipolytic bacteria in bouillons containing various percentages of salt were investigated by Hammer and Collins (31) Twenty-three cultures representing the following species were studied *Achromobacter connii*, *Achromobacter lipolyticum*, *Pseudomonas acidiconcoquens*, *P fluorescens*, *P fragi*,

Pseudomonas mucidolens, *Pseudomonas schuylkilliensis*, *Pseudomonas synxantha* and an unidentified species of *Micrococcus* Nineteen of the cultures grew in 5 per cent salt bouillon, twelve in 6 25 per cent, two in 7 5 per cent, while one (the micrococcus) grew in 12 per cent

Hof (37) studied the salt tolerances of organisms by inoculating garden soil into enrichment media containing various percentages of salt In this manner cultures of lactic acid and *Escherichia-Aerobacter* organisms were obtained in media containing 6 per cent salt, while butyric acid, urea and proteolytic bacteria were obtained in media containing 24 per cent salt Cultures from the enrichment media tolerated higher percentages of salt than laboratory cultures of the same species

Kanunnikowa (47) reported that 1 per cent salt in a medium containing 4 per cent butter favored growth of *S marcescens* and inhibited growth of *P fluorescens* Higher salt concentrations retarded the decomposition of butter Salt retarded the formation and activity of bacterial proteases and lipases

The organism which Hiscox (35) found responsible for dark discoloration in salted butter required 1 to 2 per cent salt for growth in laboratory media but was inhibited by 5 per cent White (111) reported that *P nigrifaciens* grew well in laboratory media containing 1 5 per cent salt but not at all in media containing no salt, growth was decreased by 7 5 per cent salt and was extremely slight with 10 per cent

Garrard and Lochhead (20) indicated that gram negative micrococci were less salt tolerant than gram positive micrococci There was little difference between gram negative and gram positive rods, considered as groups Many organisms isolated on 5 per cent salt agar from a salt free environment tolerated relatively high salt concentrations, over one-half growing in media containing 0 to 15 per cent salt and one-fifth growing in media containing up to 20 per cent, 25 per cent salt was definitely inhibitory to organisms isolated on a 5 per cent salt medium

Garrison (21) found that addition of 6 per cent salt to beef-extract peptone broth prevented growth of some cultures of fluorescent bacteria but not of others, only a few cultures grew

in broth containing 8 per cent salt In experimental unsalted butter made without butter culture nearly all the organisms studied produced off flavors at 21 C Addition of 2 per cent salt to the butter prevented development of off flavors by some cultures but not by others

Long and Hammer (57) reported that *P putrefaciens* grew in milk containing 4 per cent salt but not in milk containing 10 per cent, with 6 or 8 per cent salt, some cultures of the organism grew and some did not

Salt distribution Rahn (81) observed that not all water droplets in butter have the same salt content since salt is added after washing when many small droplets are already enclosed in fat He suggested that this probably is of great importance from the standpoint of the growth of microorganisms in salted butter Weigmann (110) emphasized the same ideas

Claydon and Hammer (7) concluded that salt is not entirely effective in inhibiting the putrid defect in butter unless combined with thorough working of the butter When butter containing a pure culture of *P putrefaciens* was worked only slightly, neither 1 nor 2 per cent salt prevented the defect, with thorough working either amount controlled it

Hoecker (36) used a micro procedure for determining the salt in approximately 0 2 mg portions of butter With both normal and abnormal commerical butter, some churnings had the salt very uniformly distributed while others had it poorly distributed With most churnings there was a correlation between salt distribution and incorporation of water As the working process continued, the salt became more uniformly distributed Printing butter in equipment having a reworking action did not significantly affect the salt distribution

Salt concentration necessary to inhibit bacteria Orla-Jensen (68) reported that the development of microörganisms in butter is completely inhibited only when the concentration of salt in the brine reaches 25 per cent, for example with 13 per cent water and 3 3 per cent salt Later, he (70) observed that the preserving action of salt is the more pronounced the lower the percentage of water in the butter, and he again pointed out that a relatively

high concentration of salt in the brine is essential for preventing growth of all bacteria Weigmann (109) likewise indicated that the percentage of salt necessary to preserve butter depends on the water content He (110) also noted that research on the effect of salt on microorganisms is not abundant and does not always agree He suggested that salt percentages which are toxic for some species may be stimulatory for others, thus lactic acid bacteria might be killed by a salt percentage which would stimulate undesirable species Kretchmar (51) stated that, according to its concentration in solution, the action of salt on bacteria may vary, a 0 5 molar concentration being stimulatory and a 3 0 molar concentration being inhibitory In addition, salt widens the pH range of media within which bacteria will grow

Pont (74) indicated that the concentration of salt in the moisture of butter may play a large part in controlling the activity of microörganisms, he also noted that in many experiments these concentrations have not been given and their effect on microbiological changes has not been emphasized

Hammer (30) pointed out that the influence of salt varies with the species present in butter and suggested that this is an important factor in determining whether bacterial increases occur

Relationship of initial contamination to salt effect It is probable that bacterial growth in salted butter depends to some extent on the numbers of organisms initially present Winslow, *et al* (114) reported that in broth large numbers of organisms tend to neutralize the inhibitory effect of salt and stated that there always is a mass effect caused by large numbers of living or dead cells which tends to neutralize any inhibitory action Various concentrations of *E coli*, *Micrococcus albus* and *Bacillus mesentericus* were inoculated by Slemmons (99) into bouillons containing from 1 to 12 per cent salt The larger inoculations of the organisms showed greater salt tolerance than the smaller inoculations With *M albus* an inoculation of 50 cells failed to grow in 4 per cent salt bouillon, whereas an inoculation of 50,000 cells grew in 11 per cent salt bouillon

Adaptation to salt In studying the effect of salt on the bacteria

in butter, Giltner and Baker (23) noted that the salt tolerance of some organisms could be increased by continued cultivation on salt-agar Garrard and Lochhead (20), in working with pickle brine, found that various species of bacteria can adapt themselves somewhat to changes in salt concentration and suggested that some adjustment to the high salt content of pickle brine is possible However, of 23 species investigated, all of which grew on a 5 per cent salt medium, none grew on a 10 per cent salt medium From this it was concluded that the action of 30 per cent salt involved a toxic effect, and there was no adjustment to the high salt concentration It was noted that various organisms displayed more resistance to salt in pickle brine than in broth of similar salt content Of 15 species inhibited after 5 days in 30 per cent salt broth and 22 species inhibited after 10 days, only 5 and 6 species, respectively, were inhibited in pickle brine of almost identical salt content

The available data show that salt tends to retard bacterial growth in butter and thus to delay or prevent bacterial deterioration The important factors influencing the action of salt in butter appear to be concentration in the water and uniformity of distribution throughout the water droplets Even when considerable salt is added to butter the concentration in the water may not be sufficient to inhibit all bacterial growth, and with light salting the inhibition is much less Many instances have been reported in which organisms known to be relatively sensitive to salt have produced conspicuous defects in salted butter Probably there are various causes for this, the chief one being failure to thoroughly incorporate the salt, as a result, salt-sensitive bacteria can develop in those droplets of water which contain little or no salt The present demand for lightly salted butter may account for some of the outbreaks of bacterial spoilage in butter, especially when combined with poor salt distribution The extent of contamination doubtless is of considerable practical importance in this general connection, and the ability of certain organisms to adapt themselves to higher concentrations of salt than they normally encounter also may be a factor

ACIDITY

The acid contents of different types of butter vary widely Salted butter commonly has a low acidity because of the danger of serious chemical deterioration from a combination of salt and high acid, the danger is particularly great in butters with relatively high copper content Unsalted butter made for reconstitution of cream, etc has a low acidity because of the objectionable effect of high acidity on the flavor of the reconstituted products On the other hand, unsalted butter that is intended to have a high flavor must contain considerable acid for proper flavor development Since some bacteria are known to be sensitive to acid, the acidities of certain lots of butter would be expected to influence bacterial growth therein

Orla-Jensen (68) inoculated butter with *P fluorescens* and *S lactis* and noted that the lactic acid formed by the latter organism inhibited fat hydrolysis There was little free volatile acid produced, and the odor and taste were not particularly objectionable The effect of acid production by *S lactis* on the growth of *P fluorescens* in milk was studied by Luxwolda (59) who found that both species appeared to profit by the association at 10, 13, or 15 C Since the fluorescent organism lived in the acid medium, it was believed that the milk-souring bacteria produce something besides acid which hinders the growth of fluorescent organisms in sour milk

Shutt (98) reported that churning cream at an acidity of not less than 0 35 per cent was beneficial in avoiding defects caused by *P fluorescens* since the organism grows only feebly at a pH as low as 6 6 He indicated that surface taint, which he believed could be caused by *P fluorescens*, occurs only in sweet-cream or neutralized-cream butter and never develops in sour-cream butter However, Gubitz (27) found that in bacteriological media *P fluorescens* grew at pH values as low as 5 4 to 5 8 Rahn (81) noted that the acidity of butter serum has an effect on bacterial growth in butter, but that in butter made from sour cream some organisms can grow which cannot grow in sour milk For example, sour-cream butter often becomes rancid due to growth of *P fluorescens* although this organism cannot grow in sour milk

Rahn believed that removal of some of the acid from butter during washing of the granules lowers the acidity to a point where *P fluorescens* can grow He reported that other species, such as *E coli*, *A aerogenes*, yeasts and molds, tolerate a rather highly acid medium

The pH tolerances of 505 cultures of fluorescent bacteria were investigated by Garrison (21) In beef-extract peptone broth they all grew from pH 5 5 to pH 10 0, while many grew at pH 4 5 and four grew at pH 4 0 In skim milk adjusted to pH 5 0, the 10 cultures tested grew rather rapidly The effect of butter culture on the ability of the organisms to produce defects in experimental butter was studied with 52 cultures, addition of 10 per cent butter culture to the cream prevented development of off flavors in unsalted butter by 16 of the cultures and in salted butter by 27 of them

Rogers (86) reported that during cream ripening the lactose is partly fermented to lactic and similar acids which protect the butter from fermentation by less acid-tolerant bacteria He noted that putrefactive bacteria, which often attack the curd of butter, are usually checked by acid

Mazé (62) pointed out that the bacteria causing deterioration in butter attack the casein and lactose and that these organisms are retarded by lactic acid Addition of lactic acid to the finished butter in the proportion of 0 5 to 1 g per liter was advocated as a control measure, but this was not believed to completely prevent growth

In a study of butter obtained in the Boston market, Rosenau, *et al* (90) found no relationship between numbers of bacteria and reactions of the butter Gilmour and Cruess-Callaghan (22) could detect no relationship between acidities of Irish Free State creamery butter and rates of growth of microörganisms as a group Pont and Sutton (76) reported that the majority of samples of New South Wales butter examined had pH values between 7 0 and 7 7, the modal value being 7 4 Within experimental limits, no correlation was observed between pH values and bacterial counts The data did not suggest that increased alkalinity of the serum favors development of proteolytic species

Fennema (16) found in salted sour-cream butter stored at 0 to 10 C a decrease in numbers of bacteria from the beginning of storage The decrease was rapid for the first few days, then more gradual, until at the end of several months only a few organisms remained More rapid decreases were encountered in samples originally containing large numbers of bacteria than in those originally containing small numbers In salted sweet-cream butter there was a rapid increase during the first few days of storage and then a decrease

Virtanen (107) indicated that bacteria of the water type, which often cause cheesy and putrid defects in butter, are inhibited by the acidity of sour-cream butter In studies on butter spoilage Horovitz-Vlasova, *et al* (39) noted that acidification inhibits the growth of putrefactive bacteria but may favor other organisms

Wiley (112) concluded that at 4 4 and 18 3 C butter deterioration caused by bacterial action was not delayed by the presence of acid Jacobsen (44) found that at room temperature flavor deteriorations and increases in bacteria were more extensive in unsalted non-culture butter than in unsalted culture butter

The effect of butter culture on development of surface taint in experimental butter was investigated by Derby and Hammer (10) When 10 per cent butter culture was added to pasteurized cream inoculated just before churning with surface taint butter or *P putrefaciens*, development of a putrid condition was prevented in either salted or unsalted butter held at 15 6 C *P putrefaciens* developed in milk with acidities of 0 27, 0 28 and 0 29 per cent but not in milk with acidities of 0 30 and 0 31 per cent Claydon and Hammer (7) studied the effect of pH on production of the putrid defect in unsalted butter by *P putrefaciens* and concluded that the organism can cause the defect over a wide pH range Butter made from cream with a pH of 4 5 did not spoil, that made from cream with pH values of 5 2 and 6 0 was slightly defective in 1 day at 21 C , pH values from 6 0 to 7 8 allowed rapid growth of the organism in butter The inhibitory effect of butter culture in unsalted butter also was investigated At 21 C , 5 per cent butter culture in the cream prevented the defect and was as

effective as 12 per cent, whether the organism was added to the cream before churning or to the water used to wash the butter

Pont (75) indicated that within the range of safe limits from the standpoint of chemical changes in butter, high acidities aided in minimizing the putrid defect In the work of Itzerott (43) cream acidities below 0 15 per cent had little effect on the time required for development of the putrid defect in butter Above 0 15 per cent, however, acidity appeared to have a retarding influence High acidities in conjunction with low temperatures definitely inhibited the defect Unsalted butter from cream with an acidity of 0 20 per cent and inoculated with rabbito organisms showed no evidence of the defect when held between 4 4 and 12 8 C for 3 weeks However, at higher temperatures (26 7 to 32 2 C) the taint developed despite the high acidity

Long and Hammer (57) investigated the resistance of *P putrefaciens* to various amounts of lactic acid in skim milk At a pH of approximately 5 3, the organism survived only a relatively short time, usually less than 48 hours With pH values appreciably above 5 3, it multiplied in the acidified milk, and with values below 5 3 it was killed in less than 48 hours, in one lot of milk acidified to pH 4 9, *P putrefaciens* was killed in 8 hours The authors suggested that use of butter culture in making butter should have a protective action so far as the putrid defect is concerned

In an investigation of the cheesy defect of butter, Turgasen (106) found that varying the acidity of the cream, so that the pH of the butter serum ranged from 5 4 to 7 8, did not control the spoilage, the source of infection of the butter was the wash water

Rahn and Boysen (82) showed that in unsalted butter acid-producing bacteria develop more acid in unwashed butter than in washed butter This could not be demonstrated in salted butter because of lack of acid production in the presence of salt The authors indicated that the smallest water droplets in butter are entirely enclosed in the butter granules and do not come in contact with the wash water From the outsides of the granules, however, the wash water removes all milky material since butter usually is washed until the water drains clear The water in-

corporated during the washing and working processes forms the large droplets found in butter, making in effect two entirely different types of water droplets in butter Since calculation showed that most bacteria are present in large droplets which are chiefly clear water, as compared to the small droplets, the result of washing is removal of nutrient materials For this reason washing protects the keeping quality of the butter, provided the wash water is not contaminated If the water contains lipolytic bacteria, thorough washing might be detrimental for there would not be enough lactose in the water to allow significant acid formation by the lactic acid organisms

Hunziker (41) reported that formerly it was thought washing butter improved the keeping quality by removing bacterial food However, this is not borne out in commerical practice In cream ripening the cream is impregnated with lactic acid bacteria, lactic acid and its salts, these keep undesirable bacteria in check and butter from ripened cream has better keeping quality than that from unripened cream Lightly washed, unsalted butter has better keeping quality than heavily washed, unsalted butter from the same cream

Orla-Jensen (69) found that lactic-acid-producing rods multiplied much more rapidly in unwashed than in washed butter, whereas the reverse was true with lactic-acid-producing streptococci Later, he (70) stated that the best method of preventing harmful organisms from developing to any extent in butter is to wash it so thoroughly that they will not find sufficient nutrients present

White (111) reported that *P nigrifaciens* did not grow at pH 5 2 in broth or on agar, good growth occurred at pH values from 6 8 to 8 4

The effect of butter culture and lactic acid on fat hydrolysis in cream by pure cultures of lipolytic organisms was studied by Fouts (19) When butter culture was added to the cream there was definite inhibition of *Achromobacter lipolyticum*, *Alcaligenes lipolyticus* and *P fluorescens*, when lactic acid was added to the cream used to culture the organisms, all grew even with enough acid to give a titratable acidity of 1 per cent

Accumulation of fatty acids in rancid butter has a decidedly bactericidal effect on many organisms Schmidt (95) reported a rapid increase in bacteria in a sample of rancid butter, then after 20 to 40 days a decrease which continued almost to sterility of the sample Eichholz (14) observed rancid butter which after a time was free of vegetative forms and contained only a few spores Rumment (91) noted that *P fluorescens* does not tolerate the fatty acids in rancid butter

Hammer and Collins (31) studied changes in numbers of bacteria at 21 C in 11 lots of unsalted butter made from sterilized cream inoculated with pure cultures of lipolytic organisms Early in the holding large numbers of organisms were present and rancidity developed, later, the numbers of bacteria declined until at the last examination (after 15 to 20 days) the counts were relatively low The rapid decreases in numbers of bacteria in the rancid butter were considered due to accumulation of the lower fatty acids It was pointed out, however, that decreases in numbers of organisms in various dairy products, following increases responsible for defects, are common and are not limited to defects involving fat hydrolysis

While it should be recognized that acid has an effect on the growth of bacteria in butter, the protective action of acid alone may be overemphasized Certain of the organisms considered rather sensitive to acid, for example *P putrefaciens*, tolerate pH levels lower than those normally found in butter However, addition of butter culture to cream (or wash water) seems to inhibit certain organisms and limit certain defects so that the protective action of butter culture may be due to products of metabolism other than lactic acid The inhibitory effect of volatile fatty acids should be recognized, but probably with the amounts of the acids normally present in butter this factor is not significant

AIR SUPPLY

Since most of the bacteria that develop in butter are comparatively aerobic, the air content of butter is a factor affecting

bacterial growth The relatively large numbers of organisms often found at the surface of butter and the intensity of certain flavor defects there usually are attributed to the greater air supply

Lafar (52) reported that the bacterial content of the interior of butter is much lower than that of the exterior Orla-Jensen (68) compared changes in numbers of organisms at the surface and in the interior of sweet-cream butter held at 18 to 20 C for 6 weeks At 3 days and each interval thereafter, the numbers of organisms were higher at the surface than in the interior The surface layer was disagreeable after 3 days and rancid after 7 days, while the interior was not rancid after 6 weeks Gratz (24) emphasized that bacteria develop most rapidly in the outer layers of butter and, as a consequence, lipolysis is more marked and the acid content is higher there He noted that various investigators have found air normally present in butter, because of this some bacterial increase would be expected in the inner layers

Hammer and Collins (31) investigated the growth of lipolytic bacteria in the surface and subsurface portions of unsalted butter held at 21 C The bacteria commonly grew faster at the surface than in the deeper layers and early in the holding were more numerous at the surface, later, the larger numbers were sometimes found in the interior due to death of organisms at the surface

Changes in the flora of several lots of canned butter held at room temperature were investigated by Rogers (86) Initially the flora was made up of lactic acid bacteria, yeasts and a few liquefying bacteria Both the lactic acid bacteria and yeasts decreased rapidly until at the end of 100 days only a few spore forming bacteria remained, most of which were liquefiers

Rogers (87) noted that the whitening effect of over-working butter is due to the air introduced and also that air is worked into butter in the ordinary process of manufacture He reported from 5 to 6 ml of air per 100 g of butter Rogers, *et al* (88) found that about 10 per cent by volume of fresh butter was gas, this consisted, among other things, of 20 per cent oxygen The oxygen content was materially decreased after 13 months at

−17 9 C Over-working butter in a churn did not appear to incorporate more air, it was noted that this cannot be compared to over-working small amounts of butter with a spatula Sommer and Smit (100) stated that over-working butter may increase the air content

The pore space in fresh butter was found by Pickerill and Guthrie (72) to range from 0 5 to over 6 per cent Rahn and Mohr (84) reported that the average air content of 290 samples of butter was 4 2 ml per 100 g, the variations being from 0 97 to 8 38 ml Individual dairies tended to produce butter of rather uniform air content although the content varied somewhat with the season, being high from June to October In fairly well worked butter Guthrie (28) found from 2 85 to 6 42 ml of air per 100 g, with an average of 4 65 ml, in thoroughly worked butter there was from 4 17 to 6 70 ml, with an average of 5 37 ml

Dyer (13) studied changes in the gas content of butter during storage and found that after 6 months at −17 8 C the composition of the gas in a churning of pasteurized sweet-cream butter, known to contain bacteria, showed little or no variation from the original A portion of the same churning held at 0 C showed a decided change which was characterized by a decrease in oxygen and an increase in carbon dioxide The change was further increased by holding at room temperature

Commonly the numbers of bacteria are greater at the surface of butter than in the interior This suggests that the somewhat restricted air supply in the interior may affect the growth of certain bacteria, however, growth is not prevented at favorable temperatures since the amounts of air worked into butter at the time of manufacture are sufficient for extended bacterial development

MISCELLANEOUS FACTORS

The effects of certain miscellaneous factors have been considered in the studies on bacterial changes in butter

Gases Hunziker (40) reported that carbonating does not destroy the bacteria present in cream which are harmful to the

flavor and keepmg quahty of butter made from ıt, carbonated butter from unpasteurızed cream developed the usual bacterıal flavor defects Commercıal carbon dıoxıde was found by Prescott and Parker (77) to be practıcally sterıle Butter churned ın an atmosphere of carbon dıoxıde had a lower bacterıal content than butter churned ın aır, however, buttermılk from the carbonated churnıng contaıned larger numbers of bacterıa than buttermılk from the control churnıng

The effects of oxygen, hydrogen, nıtrogen and carbon dıoxıde on growth of organısms were ınvestıgated by Prucha, *et al* (79) After a few trıals all the gases except carbon dıoxıde were dıscontınued because of unfavorable results Carbonatıon of sweet cream tended to suppress certaın types of bacterıa but dıd not hınder others, the effect was to delay sourıng of the cream for a few hours at room temperature and several days at 1 7 C When carbon dıoxıde was applıed at churnıng tıme, by chargıng the cream ın the churn and by replacıng the aır above the cream ın the churn, no sıgnıficant benefit resulted, and the fresh butter tasted sour In the butter, molds were not ınhıbıted and bacterıa were not measurably affected However, storıng butter ın an atmosphere of carbon dıoxıde ınhıbıted mold growth and prolonged keepıng qualıty In addıtıonal studıes, the same ınvestıgators (80) arrıved at essentıally the same conclusıons

Sherwood and Martın (97) reported that bacterıal counts on butter made from cream treated wıth carbon dıoxıde before churnıng dıd not ındıcate that the gas reduced the numbers of organısms present In no case was there any great dıfference between bacterıal counts on carbonated and uncarbonated butter, although varıous modıficatıons ın the manufacturıng procedure were used Erbacher and Schoppmeyer (15) found that butter stored ın an atmosphere of carbon dıoxıde developed an off flavor due to absorptıon of the gas, whıle storage ın atmospheres of hydrogen or nıtrogen accelerated putrefactıon

Preservatıves Although use of specıal preservatıves ın butter ıs now rare, they were employed extensıvely at one tıme The compounds varıed ın nature, ıt appears that some of them were ınferıor to salt

Fischer and Gruenert (18) compared various conservation materials used to increase the keeping quality of butter These included salt, benzoic acid, hydrin (a preservative analyzing 13 67 per cent free benzoic acid, 9 16 per cent sodium oxide, 7 52 per cent phosphorus pentoxide, 35 14 per cent salt, 9 26 per cent lactic acid and 24 89 per cent water), salicylic acid and boric acid Salt was considered to be much superior to the other compounds investigated

Orla-Jensen (70) reported that, where permissible by law, it is advisable to add to butter 0 75 per cent benzoic acid, 2 per cent sodium benzoate or a mixture of 0 5 per cent benzoic acid and 0 5 per cent sodium benzoate Benzoic acid was regarded as one of the less objectionable preservatives since it is transformed in the human body to hippuric acid

Use of boron preservatives was investigated in New Zealand (1) In seven experiments in which the amounts of preservatives ranged from 0 08 to 0 99 per cent, the average quality of the butter was only slightly in favor of the preserved product It was concluded that the preservatives had little effect in sustaining keeping quality, either in cold storage or afterwards at room temperature

Weigmann (110) reported that in various countries it formerly was the practice to treat butter with different conservation materials, such as boric acid preparations, sugar, saltpeter and gum arabic, he also reported that fluorides have been detected in French butter

Treatment of wash water Since contaminated wash water has been the cause of many outbreaks of defective butter, various treatments have been proposed for creamery water With some of these, such as pasteurization, no inhibitory effect would be expected to carry over into the butter, but with others involving addition of some bactericidal material to the water the substance may influence bacterial growth in the butter itself

Salmon (92) recommended that water used to wash butter be purified with ozone and reported that this treatment preserved the natural flavor of butter and retarded rancidity Dornic and Daire (12) sterilized wash water for butter with ultraviolet light and considered the process a practical one

Demeter and Haase (11) investigated the effect of washing butter with water treated by the katadyn process In fresh butter there was considerable reduction in numbers of organisms which grew on lactose agar and on casein agar and also of acid-forming organisms, no effect was noted on proteolytic or *Escherichia-Aerobacter* species When the washed butter was held in cold storage, there was no action on proteolytic organisms Butter washed with water treated too strongly developed a metallic taste

Sorensen (101) pointed out that creameries located in large cities constantly use water which contains 0 1 to 0 5 p p m of available chlorine, when water containing 5 p p m of available chlorine was used to wash butter no chlorine could be detected in the water as it left the churn Hunziker (41) reported that wash water for butter may be sterilized by treatment with 25 to 35 p p m of available chlorine and indicated that this amount has no objectionable effect on the flavor of butter

Various outbreaks of bacterial spoilage in butter have been controlled by treatment of the wash water Jensen (46) described an unusual condition which was effectively prevented by chlorination of water and heat treatment of utensils It involved variations in the quality of butter from one part to another of the same box The property chiefly affected was the flavor and, in well-marked cases, areas having a distinct off flavor were found interspersed with areas of normal flavor The defect was not noted in freshly made butter, but in butter held at 4 4 to 10 C or higher it developed rapidly, becoming noticeable in 3 to 4 days It was attributed to localized action of microorganisms carried to surfaces of the butter by wash water and utensils used in the packing operation

Ultraviolet light and x-rays Dornic and Daire (12) stated that butter cannot be sterilized by ultraviolet light on account of its opacity and because of the production of a tallowy taste and odor by the ozone generated by the lamps A method for increasing the keeping quality of butter by irradiating with x-rays for 10 minutes and storing in an atmosphere of carbon dioxide was proposed by Pimenov (73) Irradiation with ultraviolet light for

5 minutes permitted even longer keeping Contrary to the results of Dornic and Daire (12), the latter treatment did not affect the organoleptic properties of the butter

TABLE 1

Factors limiting bacterial growth in butter and suggested counterbalancing influences

FACTORS LIMITING GROWTH	SUGGESTED COUNTERBALANCING INFLUENCES
Fine dispersion of water	High initial contamination resulting in many infected water droplets Printing butter under conditions tending to aggregate water droplets Action of salt in tending to aggregate water droplets
Low temperature	Presence of psychrophilic organisms which grow just above freezing point of water Exposure of butter for short periods (during handling, etc) to temperatures allowing rapid growth Depression of freezing point of water in butter by salt
Addition of salt	Poor distribution of salt Relatively little salt added High initial contamination Presence of salt-tolerant species Adaptation of certain species to relatively high salt concentration
Use of butter culture, acidity	*S lactis* less salt-tolerant than many other species Inhibition of *S lactis* but not psychrophilic species by holding temperatures Acid content of salted butter and some unsalted butter commonly too low to inhibit organisms
Air supply	Air incorporated in butter during manufacture sufficient for many species
Special preservatives	Some that have been suggested not as effective as salt
Treatment of wash water	Outbreaks of defective butter attributed to contaminated wash water actually due to some other cause

GENERAL CONSIDERATIONS

From the studies reported in the literature it is evident that there are various factors which tend to restrain the growth of

bacteria in butter The data suggest that some of these are very effective if they are completely operative, and the combined action of two or more of them would be expected to control bacterial action However, under commercial conditions bacterial spoilage of butter occurs rather frequently, even when protective measures are employed in the manufacture This spoilage indicates that there are influences tending to counterbalance the restraining actions When bacterial growth occurs in butter it is probable that more than one of these counterbalancing influences are involved In general the growth of bacteria in butter must be considered from the standpoint of the balance between the factors restraining growth and those favoring growth, in this balance the effect of the extent of contamination is frequently overlooked

The factors limiting bacterial growth in butter and suggested counterbalancing influences are listed in table 1

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